

05-24-00

A

05/23/00  
jc822 U.S. PRO

jc831 U.S. PRO  
09/576290  
06/23/00

Practitioner's Docket No. 47176-DIV (342)

PATENT

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Box Patent Application  
Assistant Commissioner for Patents  
Washington, D.C. 20231

NEW APPLICATION TRANSMITTAL

Transmitted herewith for filing is the patent application of

Inventor(s): Shuji HINUMA, Yugo HABATA, Yuji KAWAMATA, Masaki HOSOYA,  
Ryo FUJII, Shoji FUKUSUMI and Chieko KITADA

WARNING: 37 CFR 1.41(a)(1) points out:

"(a) A patent is applied for in the name or names of the actual inventor or inventors.

(1) The inventorship of a nonprovisional application is that inventorship set forth in the oath or declaration as prescribed by § 1.63, except as provided for in § 1.53(d)(4) and § 1.63(d). If an oath or declaration as prescribed by § 1.63 is not filed during the pendency of a nonprovisional application, the inventorship is that inventorship set forth in the application papers filed pursuant to § 1.53(b), unless a petition under this paragraph accompanied by the fee set forth in § 1.17(i) is filed supplying or changing the name or names of the inventor or inventors."

For (title): POLYPEPTIDES, THEIR PRODUCTION AND USE

CERTIFICATION UNDER 37 C.F.R. 1.10\*

(Express Mail label number is **mandatory**.)

(Express Mail certification is optional.)

I hereby certify that this correspondence and the documents referred to as attached therein are being deposited with the United States Postal Service on this date May 23, 2000, in an envelope as "Express Mail Post Office to Addressee," mailing Label Number EL180585522US, addressed to the: Assistant Commissioner for Patents, Washington, D.C. 20231.

Susan M. Dillon  
(type or print name of person mailing paper)

Susan M Dillon  
Signature of person mailing paper

WARNING: Certificate of mailing (first class) or facsimile transmission procedures of 37 C.F.R. 1.8 cannot be used to obtain a date of mailing or transmission for this correspondence.

\*WARNING: Each paper or fee filed by "Express Mail" **must** have the number of the "Express Mail" mailing label placed thereon prior to mailing. 37 C.F.R. 1.10(b).  
"Since the filing of correspondence under § 1.10 without the Express Mail mailing label thereon is an oversight that can be avoided by the exercise of reasonable care, requests for waiver of this requirement will **not** be granted on petition." Notice of Oct. 24, 1996, 60 Fed. Reg. 56,439, at 56,442.

## 1. Type of Application

This new application is for a(n)

(check one applicable item below)

☒ Original (nonprovisional)

☐ Design

☐ Plant

**WARNING:** *Do not use this transmittal for a completion in the U.S. of an International Application under 35 U.S.C. 371(c)(4), unless the International Application is being filed as a divisional, continuation or continuation-in-part application.*

**WARNING:** *Do not use this transmittal for the filing of a provisional application.*

**NOTE:** *If one of the following 3 items apply, then complete and attach ADDED PAGES FOR NEW APPLICATION TRANSMITTAL WHERE BENEFIT OF A PRIOR U.S. APPLICATION CLAIMED and a NOTIFICATION IN PARENT APPLICATION OF THE FILING OF THIS CONTINUATION APPLICATION.*

☒ Divisional.

☐ Continuation.

☐ Continuation-in-part (C-I-P).

## 2. Benefit of Prior U.S. Application(s) (35 U.S.C. 119(e), 120, or 121)

**NOTE:** *A nonprovisional application may claim an invention disclosed in one or more prior filed copending nonprovisional applications or copending international applications designating the United States of America. In order for a nonprovisional application to claim the benefit of a prior filed copending nonprovisional application or copending international application designating the United States of America, each prior application must name as an inventor at least one inventor named in the later filed nonprovisional application and disclose the named inventor's invention claimed in at least one claim of the later filed nonprovisional application in the manner provided by the first paragraph of 35 U.S.C. 112. Each prior application must also be:*

*(i) An international application entitled to a filing date in accordance with PCT Article 11 and designating the United States of America; or*

*(ii) Complete as set forth in § 1.51(b); or*

*(iii) Entitled to a filing date as set forth in § 1.53(b) or § 1.53(d) and include the basic filing fee set forth in § 1.16; or*

*(iv) Entitled to a filing date as set forth in § 1.53(b) and have paid therein the processing and retention fee set forth in § 1.21(l) within the time period set forth in § 1.53(f).*

37 CFR 1.78(a)(1).

**NOTE** *If the new application being transmitted is a divisional, continuation or a continuation-in-part of a parent case, or where the parent case is an International Application which designated the U.S., or benefit of a prior provisional*

application is claimed, then check the following item and complete and attach ADDED PAGES FOR NEW APPLICATION TRANSMITTAL WHERE BENEFIT OF PRIOR U.S. APPLICATION(S) CLAIMED.

**WARNING:** If an application claims the benefit of the filing date of an earlier filed application under 35 U.S.C. 120, 121 or 365(c), the 20-year term of that application will be based upon the filing date of the earliest U.S. application that the application makes reference to under 35 U.S.C. 120, 121 or 365(c). (35 U.S.C. 154(a)(2) does not take into account, for the determination of the patent term, any application on which priority is claimed under 35 U.S.C. 119, 365(a) or 365(b).) For a c-i-p application, applicant should review whether any claim in the patent that will issue is supported by an earlier application and, if not, the applicant should consider canceling the reference to the earlier filed application. The term of a patent is not based on a claim-by-claim approach. See Notice of April 14, 1995, 60 Fed. Reg. 20,195, at 20,205.

**WARNING:** When the last day of pendency of a provisional application falls on a Saturday, Sunday, or Federal holiday within the District of Columbia, any nonprovisional application claiming benefit of the provisional application **must** be filed prior to the Saturday, Sunday, or Federal holiday within the District of Columbia. See 37 C.F.R. § 1.78(a)(3).

☒ The new application being transmitted claims the benefit of prior U.S. application(s).  
Enclosed are ADDED PAGES FOR NEW APPLICATION TRANSMITTAL WHERE  
BENEFIT OF PRIOR U.S. APPLICATION(S) CLAIMED.

### 3. Papers Enclosed

#### A. Required for Filing Date under 37 C.F.R. 1.53(b) (Regular) or 37 C.F.R. 1.153 (Design) Application

157 Pages of Specification  
3 Pages of Claims  
53 Sheets of Drawing

☒ Formal  
☐ Informal

#### B. Other Papers Enclosed

1 Pages of Abstract  
\_\_\_\_\_ Other

**WARNING:** **DO NOT** submit original drawings. A high quality copy of the drawings should be supplied when filing a patent application. The drawings that are submitted to the Office must be on strong, white, smooth, and non-shiny paper and meet the standards according to § 1.84. If corrections to the drawings are necessary, they should be made to the original drawing and a high-quality copy of the corrected original drawing then submitted to the Office. Only one copy is required or desired. For comments on proposed then-new 37 C.F.R. 1.84, see Notice of March 9, 1988 . . . (1990 O.G. 57-62).

**NOTE:** "Identifying indicia, if provided, should include the application number or the title of the invention, inventor's name, docket number (if any), and the name and telephone number of a person to call if the Office is unable to match the drawings to the proper application. This information should be placed on the back of each sheet of drawing a minimum distance of 1.5 cm. (5/8 inch) down from the top of the page." 37 C.F.R. 1.84(c)).

(complete the following, if applicable)

- ☐ The enclosed drawing(s) are photograph(s), and there is also attached a "PETITION TO ACCEPT PHOTOGRAPH(S) AS DRAWING(S)." 37 C.F.R. 1.84(b).

#### 4. Additional Papers Enclosed

- ☒ Preliminary Amendment  
☐ Information Disclosure Statement (37 C.F.R. 1.98)  
☐ Form PTO-1449  
☐ Citations  
☐ Declaration of Biological Deposit  
☒ Submission of "Sequence Listing," computer readable copy and/or amendment pertaining thereto for biotechnology invention containing nucleotide and/or amino acid sequence.  
☐ Authorization of Attorney(s) to Accept and Follow Instructions from Representative  
☐ Special Comments  
☐ Other:

#### 5. Declaration or Oath

*NOTE: A newly executed declaration is not required in a continuation or divisional application provided the prior nonprovisional application contained a declaration as required, the application being filed is by all or fewer than all the inventors named in the prior application, there is no new matter in the application being filed, and a copy of the executed declaration filed in the prior application (showing the signature or an indication thereon that it was signed) is submitted. The copy must be accompanied by a statement requesting deletion of the names of person(s) who are not inventors of the application being filed. If the declaration in the prior application was filed under § 1.47 then a copy of that declaration must be filed accompanied by a copy of the decision granting § 1.47 status or, if a nonsigning person under § 1.47 has subsequently joined in a prior application, then a copy of the subsequently executed declaration must be filed. See 37 CFR 1.63(d).*

*NOTE: A declaration filed to complete an application must be executed, identify the specification to which it is directed, identify each inventor by full name, including the family name, and at least one given name without abbreviation together with any other given name or initial, and the residence, post office address and country of citizenship of each inventor and state whether the inventor is a sole or joint inventor. 37 CFR 1.63(a)(1)-(4).*

- ☒ Enclosed (Copy from parent application)

Executed by

(check all applicable boxes)

- ☒ inventor(s).  
☐ legal representative of inventor(s). 37 CFR 1.42 or 1.43.  
☐ joint inventor or person showing a proprietary interest on behalf of inventor who refused to sign or cannot be reached.  
☐ This is the petition required by 37 CFR 1.47 and the statement required by 37 CFR 1.47 is also attached. See item 13 below for fee.  
☐ Not Enclosed.

*NOTE: Where the filing is a completion in the U.S. of an International Application, or where the completion of the U.S. application contains subject matter in addition to the International Application, the application may be treated as a continuation or continuation-in-part, as the case may be, utilizing ADDED PAGE FOR NEW APPLICATION TRANSMITTAL WHERE BENEFIT OF PRIOR U.S. APPLICATION CLAIMED.*



- ☐ Application is made by a person authorized under 37 C.F.R. 1.41(c) on behalf of all the above named inventor(s).

*(The declaration or oath, along with the surcharge required by 37 CFR 1.16(e), can be filed subsequently).*

NOTE: It is important that all the correct inventor(s) are named for filing under 37 CFR 1.41(c) and 1.53(b).

- ☐ Showing that the filing is authorized.  
(not required unless called into question. 37 CFR 1.41(d))

## 6. Inventorship Statement

**WARNING:** If the named inventors are each not the inventors of all the claims an explanation, including the ownership of the various claims at the time the last claimed invention was made, should be submitted.

The inventorship for all the claims in this application are:

- ☐ The same.
- or
- ☐ Not the same. An explanation, including the ownership of the various claims at the time the last claimed invention was made,
- ☐ is submitted.
- ☐ will be submitted.

## 7. Language

NOTE: An application including a signed oath or declaration may be filed in a language other than English. An English translation of the non-English language application and the processing fee of \$130.00 required by 37 CFR 1.17(k) is required to be filed with the application, or within such time as may be set by the Office. 37 CFR 1.52(d).

- ☒ English
- ☐ Non-English

- ☐ The attached translation includes a statement that the translation is accurate. 37 C.F.R. 1.52(d).

## 8. Assignment

- ☒ An assignment of the invention to Takeda Chemical Industries, Ltd.
- ☐ is attached. A separate ☐ "COVER SHEET FOR ASSIGNMENT (DOCUMENT) ACCOMPANYING NEW PATENT APPLICATION" or ☐ FORM PTO 1595 is also attached.
- ☒ was filed in the parent application
- ☐ will follow.

NOTE: "If an assignment is submitted with a new application, send two separate letters-one for the application and one for the assignment" Notice of May 4, 1990 (1114 O.G. 77-78).

**WARNING:** A newly executed "STATEMENT UNDER 37 CFR 3.73(b)" must be filed when a continuation-in-part application is filed by an assignee. Notice of April 30, 1993, 1150 O.G. 62-64.

**9. Certified Copy**

Certified copy(ies) of application(s)

<u>Country</u>	<u>Appln. No.</u>	<u>Filed</u>
----------------	-------------------	--------------

from which priority is claimed

- ☐ is enclosed.  
☐ was filed.  
☐ will follow.

NOTE: The foreign application forming the basis for the claim for priority must be referred to in the oath or declaration. 37 CFR 1.55(a) and 1.63.

NOTE: This item is for any foreign priority for which the application being filed directly relates. If any parent U.S. application or International Application from which this application claims benefit under 35 U.S.C. 120 is itself entitled to priority from a prior foreign application, then complete item 18 on the ADDED PAGES FOR NEW APPLICATION TRANSMITTAL WHERE BENEFIT OF PRIOR U.S. APPLICATION(S) CLAIMED.

**10. Fee Calculation (37 C.F.R. 1.16)**

A. ☒ Regular application

---

**CLAIMS AS FILED**

---

Claims	Number Filed	Basic Fee Allowance	Number Extra	Rate	Basic Fee 37 C.F.R. 1.16(a) \$690.00
<b>Total Claims</b> (37 CFR 1.16(c))	7	- 20 =	0	x \$ 18.00	\$0
<b>Independent Claims</b> (37 CFR 1.16(b))	3	- 3 =	0	x \$78.00	\$0
<b>Multiple Dependent Claim(s), if any</b> (37 CFR 1.16(d))			+	\$260.00	\$0

- ☐ Amendment cancelling extra claims is enclosed.  
☐ Amendment deleting multiple-dependencies is enclosed.  
☐ Fee for extra claims is not being paid at this time.

NOTE: If the fees for extra claims are not paid on filing they must be paid or the claims cancelled by amendment, prior to the expiration of the time period set for response by the Patent and Trademark Office in any notice of fee deficiency. 37 CFR 1.16(d).

Filing Fee Calculation \$ 690.00

B. ☐ Design application  
(\$330.00—37 CFR 1.16(f))

Filing Fee Calculation \$ \_\_\_\_\_

C. ☐ Plant application  
(\$540.00—37 CFR 1.16(g))

Filing Fee Calculation \$ \_\_\_\_\_

# 11. Small Entity Statement(s)

☐ Statement(s) that this is a filing by a small entity under 37 CFR 1.9 and 1.27 is (are) attached.

**WARNING:** "Status as a small entity must be specifically established in each application or patent in which the status is available and desired. Status as a small entity in one application or patent does not affect any other application or patent, including applications or patents which are directly or indirectly dependent upon the application or patent in which the status has been established. The refiling of an application under § 1.53 as a continuation, division, or continuation-in-part (including a continued prosecution application under § 1.53(d)), or the filing of a reissue application requires a new determination as to continued entitlement to small entity status for the continuing or reissue application. A nonprovisional application claiming benefit under 35 U.S.C. 119(e), 120, 121, or 365(c) of a prior application, or a reissue application may rely on a statement filed in the prior application or in the patent if the nonprovisional application or the reissue application includes a reference to the statement in the prior application or in the patent or includes a copy of the statement in the prior application or in the patent and status as a small entity is still proper and desired. The payment of the small entity basic statutory filing fee will be treated as such a reference for purposes of this section." 37 CFR 1.28(a)(2).

(complete the following, if applicable)

☐ Status as a small entity was claimed in prior application \_\_\_\_\_, filed on \_\_\_\_\_ from which benefit is being claimed for this application under:

35 U.S.C. § ☐ 119(e),  
☐ 120,  
☐ 121,  
☐ 365(c),

and which status as a small entity is still proper and desired.

☐ A copy of the statement in the prior application is included.

Filing Fee Calculation (50% of A, B or C above) \$ \_\_\_\_\_

NOTE: Any excess of the full fee paid will be refunded if a small entity status is established refund request are filed within 2 months of the date of timely payment of a full fee. The two-month period is not extendable under § 1.136. 37 CFR 1.28(a).

12. **Request for International-Type Search** (37 C.F.R. 1.104(d))

(complete, if applicable)

- ☐ Please prepare an international-type search report for this application at the time when national examination on the merits takes place.

13. **Fee Payment Being Made at This Time**

- ☐ Not Enclosed

- ☐ No filing fee is to be paid at this time.  
(This and the surcharge required by 37 C.F.R. 1.16(e) can be paid subsequently.)

- ☒ Enclosed

- ☒ Filing fee \$ 690.00

- ☐ Recording assignment  
(\$40.00; 37 C.F.R. 1.21(h))  
(See attached "COVER SHEET FOR  
ASSIGNMENT ACCOMPANYING NEW  
APPLICATION.") \$ \_\_\_\_\_

- ☐ Petition fee for filing by other than  
all the inventors or person on behalf  
of the inventor where inventor  
refused to sign or cannot be reached  
(\$130.00; 37 C.F.R. 1.47 and 1.17(i)) \$ \_\_\_\_\_

- ☐ For processing an application with a  
specification in a non-English language  
(\$130.00; 37 C.F.R. 1.52(d) and 1.17(k)) \$ \_\_\_\_\_

- ☐ Processing and retention fee  
(\$130.00; 37 C.F.R. 1.53(d) and 1.21(l)) \$ \_\_\_\_\_

- ☐ Fee for international-type search report  
(\$40.00; 37 C.F.R. 1.21(e)) \$ \_\_\_\_\_

NOTE: 37 CFR 1.21(l) establishes a fee for processing and retaining any application that is abandoned for failing to complete the application pursuant to 37 CFR 1.53(f) and this, as well as the changes to 37 CFR 1.53 and 1.78(a)(1), indicate that in order to obtain the benefit of a prior U.S. application, either the basic filing fee must be paid, or the processing and retention fee of § 1.21(l) must be paid, within 1 year from notification under § 53(f).

Total Fees Enclosed \$ 690.00

**14. Method of Payment of Fees**

- ☒ Check in the amount of \$ 690.00
- ☐ Charge Account No. \_\_\_\_\_ in the amount of \$ \_\_\_\_\_.  
A duplicate of this transmittal is attached.

*NOTE: Fees should be itemized in such a manner that it is clear for which purpose the fees are paid. 37 CFR 1.22(b).*

**15. Authorization to Charge Additional Fees**

**WARNING:** *If no fees are to be paid on filing, the following items should not be completed.*

**WARNING:** *Accurately count claims, especially multiple dependent claims, to avoid unexpected high charges, if extra claim charges are authorized.*

- ☒ The Commissioner is hereby authorized to charge the following additional fees by this paper and during the entire pendency of this application to Account No. 04-1105.
- ☒ 37 C.F.R. 1.16(a), (f) or (g) (filing fees)
- ☒ 37 C.F.R. 1.16(b), (c) and (d) (presentation of extra claims)

*NOTE: Because additional fees for excess or multiple dependent claims not paid on filing or on later presentation must only be paid or these claims cancelled by amendment prior to the expiration of the time period set for response by the PTO in any notice of fee deficiency (37 CFR 1.16(d)), it might be best not to authorize the PTO to charge additional claim fees, except possibly when dealing with amendments after final action.*

- ☒ 37 C.F.R. 1.16(e) (surcharge for filing the basic filing fee and/or declaration on a date later than the filing date of the application)
- ☒ 37 CFR 1.17(a)(1)-(5) (extension fees pursuant to § 1.136(a).
- ☒ 37 C.F.R. 1.17 (application processing fees)

*NOTE: "A written request may be submitted in an application that is an authorization to treat any concurrent or future reply, requiring a petition for an extension of time under this paragraph for its timely submission, as incorporating a petition for extension of time for the appropriate length of time. An authorization to charge all required fees, fees under § 1.17, or all required extension of time fees will be treated as a constructive petition for an extension of time in any concurrent or future reply requiring a petition for an extension of time under this paragraph for its timely submission. Submission of the fee set forth in § 1.17(a) will also be treated as a constructive petition for an extension of time in any concurrent reply requiring a petition for an extension of time under this paragraph for its timely submission." 37 CFR 1.136(a)(3).*

- ☐ 37 C.F.R. 1.18 (issue fee at or before mailing of Notice of Allowance, pursuant to 37 C.F.R. 1.311(b))

*NOTE: Where an authorization to charge the issue fee to a deposit account has been filed before the mailing of a Notice of Allowance, the issue fee will be automatically charged to the deposit account at the time of mailing the notice of allowance. 37 CFR 1.311(b)).*

*NOTE: 37 CFR 1.28(b) requires "Notification of any change in status resulting in loss of entitlement to small entity status must be filed in the application . . . prior to paying, or at the time of paying, . . . issue fee." From the wording of 37 CFR 1.28(b), (a) notification of change of status must be made even if the fee is paid as "other than a small entity" and (b) no notification is required if the change is to another small entity.*

16. Instructions as to Overpayment

NOTE: "... Amounts of twenty-five dollars or less will not be returned unless specifically requested within a reasonable time, nor will the payer be notified of such amounts; amounts over twenty-five dollars may be returned by check or, if requested, by credit to a deposit account." 37 CFR 1.26(a).

☒ Credit Account No. 04-1105

☐ Refund

Christine C. O'Day  
SIGNATURE OF PRACTITIONER

Reg. No. 38,256

Christine C. O'Day  
(type or print name of practitioner)

Tel. No.: (617) 523-3400

Dike, Bronstein, Roberts & Cushman, LLP  
130 Water Street  
P.O. Address

Customer No.:

Boston, MA 02109

☒ **Incorporation by reference of added pages**

*(check the following item if the application in this transmittal claims the benefit of prior U.S. application(s) (including an international application entering the U.S. stage as a continuation, divisional or C-I-P application) and complete and attach the ADDED PAGES FOR NEW APPLICATION TRANSMITTAL WHERE BENEFIT OF PRIOR U.S. APPLICATION(S) CLAIMED)*

☒ Plus Added Pages for New Application Transmittal Where Benefit of Prior U.S. Application(s) Claimed

Number of pages added 5

☒ Plus Added Pages for Papers Referred to in Item 4 Above

Number of pages added 8

☐ Plus added pages deleting names of inventor(s) named on prior application(s) who is/are no longer inventor(s) of the subject matter claimed in this application.

Number of pages added \_\_\_\_\_

☐ Plus "Assignment Cover Letter Accompanying New Application"

Number of pages added \_\_\_\_\_

☐ **Statement Where No Further Pages Added**

*(if no further pages form a part of this Transmittal, then end this Transmittal with this page and check the following item)*

☐ This transmittal ends with this page.



# **ADDED PAGES FOR APPLICATION TRANSMITTAL WHERE BENEFIT OF PRIOR U.S. APPLICATION(S) CLAIMED**

NOTE: See 37 CFR 1.78.

## **17. Relate Back**

**WARNING:** If an application claims the benefit of the filing date of an earlier filed application under 35 U.S.C. 120, 121 or 365(c), the 20-year term of that application will be based upon the filing date of the earliest U.S. application that the application makes reference to under 35 U.S.C. 120, 121 or 365(c). (35 U.S.C. 154(a)(2) does not take into account, for the determination of the patent term, any application on which priority is claimed under 35 U.S.C. 119, 365(a) or 365(b).) For a c-i-p application, applicant should review whether any claim in the patent that will issue is supported by an earlier application and, if not, the applicant should consider canceling the reference to the earlier filed application. The term of a patent is not based on a claim-by-claim approach. See Notice of April 14, 1995, 60 Fed. Reg. 20,195, at 20,205.

*(complete the following, if applicable)*

☒ Amend the specification by inserting, before the first line, the following sentence:

### **A. 35 U.S.C. 119(e)**

NOTE: "Any nonprovisional application claiming the benefit of one or more prior filed copending provisional applications must contain or be amended to contain in the first sentence of the specification following the title a reference to each such prior provisional application, identifying it as a provisional application, and including the provisional application number (consisting of series code and serial number)." 37 C.F.R. § 1.78(a)(4).

☐ "This application claims the benefit of U.S. Provisional Application(s) No(s).:

**APPLICATION NO(S).:**

**FILING DATE**

_____	_____
_____	_____
_____ / _____	_____

### **B. 35 U.S.C. 120, 121 and 365(c)**

NOTE: "Except for a continued prosecution application filed under § 1.53(d), any nonprovisional application claiming the benefit of one or more prior filed copending nonprovisional applications or international applications designating the United States of America must contain or be amended to contain in the first sentence of the specification following the title a reference to each such prior application, identifying it by application number (consisting of the series code and serial number) or international application number and international filing date and indicating the relationship of the applications. . . . Cross-references to other related applications may be made when appropriate." (See § 1.14(a)). 37 C.F.R. § 1.78(a)(2).

☒ "This application is a



☐ continuation

☐ continuation-in-part

☒ divisional

of copending application(s)

☒ application number 08/776,971, filed 2/6/97, which is a continuation of application number PCT/JP96/03821, filed 12/28/96.

☐ International Application \_\_\_\_\_ filed on \_\_\_\_\_ and which designated the U.S.”

NOTE: The proper reference to a prior filed PCT application that entered the U.S. national phase is the U.S. serial number and the filing date of the PCT application that designated the U.S.

NOTE: (1) Where the application being transmitted adds subject matter to the International Application, then the filing can be as a continuation-in-part or (2) if it is desired to do so for other reasons then the filing can be as a continuation.

NOTE: The deadline for entering the national phase in the U.S. for an international application was clarified in the Notice of April 28, 1987 (1079 O.G. 32 to 46) as follows:

“The Patent and Trademark Office considers the International application to be pending until the 22nd month from the priority date if the United States has been designated and no Demand for International Preliminary Examination has been filed prior to the expiration of the 19th month from the priority date and until the 32nd month from the priority date if a Demand for International Preliminary Examination which elected the United States of America has been filed prior to the expiration of the 19th month from the priority date, provided that a copy of the international application has been communicated to the Patent and Trademark Office within the 20 or 30 month period respectively. If a copy of the international application has not been communicated to the Patent and Trademark Office within the 20 or 30 month period respectively, the international application becomes abandoned as to the United States 20 or 30 months from the priority date respectively. These periods have been placed in the rules as paragraph (h) of § 1.494 and paragraph (i) of § 1.495. A continuing application under 35 U.S.C. 365(c) and 120 may be filed anytime during the pendency of the international application.”

☐ “The nonprovisional application designated above, namely application \_\_\_\_\_/\_\_\_\_\_, filed \_\_\_\_\_, claims the benefit of U.S. Provisional Application(s) No(s).:

**APPLICATION NO(S):**

**FILING DATE**

_____/_____	_____”
_____/_____	_____”
_____/_____	_____”

☐ Where more than one reference is made above please combine all references into one sentence.

## 18. Relate Back—35 U.S.C. 119 Priority Claim for Prior Application

The prior U.S. application(s), including any prior International Application designating the U.S., identified above in item 17B, in turn itself claim(s) foreign priority(ies) as follows:

Country	Appln. no.	Filed
---------	------------	-------

The certified copy(ies) has (have)

☐ been filed on \_\_\_\_\_, in prior application \_\_\_\_\_, which was filed on \_\_\_\_\_.

☐ is (are) attached.

**WARNING:** The certified copy of the priority application that may have been communicated to the PTO by the International Bureau may not be relied on without any need to file a certified copy of the priority application in the continuing application. This is so because the certified copy of the priority application communicated by the International Bureau is placed in a folder and is not assigned a U.S. serial number unless the national stage is entered. Such folders are disposed of if the national stage is not entered. Therefore, such certified copies may not be available if needed later in the prosecution of a continuing application. An alternative would be to physically remove the priority documents from the folders and transfer them to the continuing application. The resources required to request transfer, retrieve the folders, make suitable record notations, transfer the certified copies, enter and make a record of such copies in the Continuing Application are substantial. Accordingly, the priority documents in folders of international applications that have not entered the national stage may not be relied on. Notice of April 28, 1987 (1079 O.G. 32 to 46).

## 19. Maintenance of Copendency of Prior Application

**NOTE:** The PTO finds it useful if a copy of the petition filed in the prior application extending the term for response is filed with the papers constituting the filing of the continuation application. Notice of November 5, 1985 (1060 O.G. 27).

A. ☐ Extension of time in prior application

(This item **must** be completed and the papers filed **in the prior application**, if the period set in the prior application has run.)

☐ A petition, fee and response extends the term in the pending **prior** application until \_\_\_\_\_

☐ A **copy** of the petition filed in prior application is attached.

B. ☐ Conditional Petition for Extension of Time in Prior Application

(complete this item, if previous item not applicable)

☐ A conditional petition for extension of time is being filed in the pending **prior** application.

☐ A **copy** of the conditional petition filed in the prior application is attached.

**20. Further Inventorship Statement Where Benefit of Prior Application(s) Claimed**

*(complete applicable item (a), (b) and/or (c) below)*

- (a) ☒ This application discloses and claims only subject matter disclosed in the prior application whose particulars are set out above and the inventor(s) in this application are

☒ the same.

☐ less than those named in the prior application. It is requested that the following inventor(s) identified for the prior application be deleted:

---

*(type name(s) of inventor(s) to be deleted)*

- (b) ☐ This application discloses and claims additional disclosure by amendment and a new declaration or oath is being filed. With respect to the prior application, the inventor(s) in this application are

☐ the same.

☐ the following additional inventor(s) have been added:

---

*(type name(s) of inventor(s) to be deleted)*

- (c) ☐ The inventorship for all the claims in this application are

☐ the same.

☐ not the same. An explanation, including the ownership of the various claims at the time the last claimed invention was made

☐ is submitted.

☐ will be submitted.

**21. Abandonment of Prior Application *(if applicable)***

- ☐ Please abandon the prior application at a time while the prior application is pending, or when the petition for extension of time or to revive in that application is granted, and when this application is granted a filing date, so as to make this application copending with said prior application.

NOTE: According to the Notice of May 13, 1983 (103, TMOG 6-7), the filing of a continuation or continuation-in-part application is a proper response with respect to a petition for extension of time or a petition to revive and should include the express abandonment of the prior application conditioned upon the granting of the petition and the granting of a filing date to the continuing application.

## 22. Petition for Suspension of Prosecution for the Time Necessary to File an Amendment

**WARNING:** "The claims of a new application may be finally rejected in the first Office action in those situations where (1) the new application is a continuing application of, or a substitute for, an earlier application, and (2) all the claims of the new application (a) are drawn to the same invention claimed in the earlier application, and (b) would have been properly finally rejected on the grounds of art of record in the next Office action if they had been entered in the earlier application." MPEP, § 706.07(b).

**NOTE:** Where it is possible that the claims on file will give rise to a first action final for this continuation application and for some reason an amendment cannot be filed promptly (e.g., experimental data is being gathered) it may be desirable to file a petition for suspension of prosecution for the time necessary.

(check the next item, if applicable)

☐ There is provided herewith a Petition To Suspend Prosecution for the Time Necessary to File An Amendment (New Application Filed Concurrently)

## 23. Small Entity (37 CFR § 1.28(a))

☐ Applicant has established small entity status by the filing of a statement in parent application No. \_\_\_\_\_.

☐ A copy of the statement previously filed is included.

**WARNING:** See 37 CFR § 1.28(a).

## 24. NOTIFICATION IN PARENT APPLICATION OF THIS FILING

☐ A notification of the filing of this  
(check one of the following)

☐ continuation

☐ continuation-in-part

☐ divisional

is being filed in the parent application, from which this application claims priority under 35 U.S.C. § 120.

**IN THE UNITED STATES PATENT AND TRADEMARK OFFICE**

APPLICANT: Hinuma et al.

U.S.S.N.: Not Yet Assigned                      GROUP: Not Yet Assigned  
[Express Mail Label No. EL180585522US; Div. of 08/776,971]

FILED: Herewith                      EXAMINER: Not Yet Assigned

FOR: POLYPEPTIDES, THEIR PRODUCTION AND USE

Honorable Commissioner of Patents and Trademarks  
Washington, D.C. 20231

Sir:

**PRELIMINARY AMENDMENT**

Applicants kindly ask that the above-identified application be amended as follows:

**IN THE CLAIMS:**

Kindly add the following new claims.

--21. (new) A labeled polypeptide which comprises an amino acid sequence represented by SEQ ID NO:73 or its amide or ester, or a salt thereof.

22. (new) The labeled polypeptide or its amide or ester, or a salt thereof, as claimed in claim 21, which is labeled with [<sup>3</sup>H], [<sup>125</sup>I], [<sup>14</sup>C] or [<sup>35</sup>S].

23. (new) The labeled polypeptide or its amide or ester, or a salt thereof, as claimed in claim 21, which is labeled with [<sup>125</sup>I].

24. (new) The labeled polypeptide as claimed in claim 21, which comprises the amino acid sequence represented by SEQ ID NO:3, SEQ ID NO:4, SEQ ID NO:5, SEQ ID NO:6, SEQ ID NO:7, SEQ ID NO:8, SEQ ID NO:9, SEQ ID NO:10, SEQ ID NO:47, SEQ ID NO:48, SEQ ID NO:49, SEQ ID NO:50, SEQ ID NO:51, SEQ ID NO:52, SEQ ID NO:61, SEQ ID NO:62, SEQ ID NO:63, SEQ ID NO:64, SEQ ID NO:65 or SEQ ID NO:66.

Hinuma et al.

U.S.S.N. Not Yet Assigned  
Express Mail Label No. EL180585522US  
Divisional of U.S.S.N. 08/776,971

Page 2

25. (new) The labeled polypeptide as claimed in claim 24, which comprises the amino acid sequence represented by SEQ ID NO:61 or SEQ ID NO:64, and C-terminus of the polypeptide is amidated.

26. (new) A labeled polypeptide which comprises the amino acid sequence represented by SEQ ID NO:61 or SEQ ID NO:64, and the C-terminus of the polypeptide is amidated and labeled with [<sup>125</sup>I].

27. (new) A polypeptide which comprises an amino acid sequence represented by SEQ ID NO:61 or SEQ ID NO:64, wherein the C-terminus of the polypeptide is amidated.--

Please cancel claims 1-20 without prejudice.

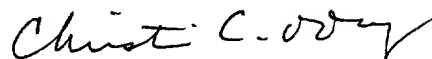
#### REMARKS

Claims 1-20 have been cancelled without prejudice and claims 21-27 have been added. No new matter is presented by virtue of this Amendment.

Applicants respectfully request entry of this Amendment prior to examination.

Early consideration and allowance of the application are earnestly solicited.

Respectfully submitted,



Christine C. O'Day, (Reg. 38,256)  
DIKE, BRONSTEIN, ROBERTS  
& CUSHMAN, LLP  
130 Water Street  
Boston, MA 02109  
Tel: (617) 523-3400  
Fax: (617) 523-6440

DESCRIPTION  
POLYPEPTIDES, THEIR PRODUCTION AND USE

[Technical Field]

5           The present invention relates to a novel ligand polypeptide for the G protein-coupled receptor protein and a DNA comprising a DNA encoding the ligand polypeptide.

10       [Background Art]

          Many hormones and neurotransmitters mediate biological functions through specific receptors present on the cell membrane. Many of these receptors engage themselves in the intracellular transduction of signals through activation of the coupled guanine nucleotide-binding protein (hereinafter sometimes referred to briefly as G protein) and have the common structure comprising 7 transmembrane domains. Therefore, these receptors are collectively referred to as G protein-coupled receptor or 7-transmembrane receptor.

          One of the pathways to modulate biological functions mediated by such hormones or neurotransmitters through G protein-coupled receptors is the hypothalamo-pituitary system. Thus, the secretion of pituitary hormone from the hypophysis is controlled by hypothalamic hormones (pituitatropic releasing factor) and the functions of the target cells or organs are regulated through the pituitary hormones released into the circulation. This pathway carries out functional modulations of importance to the living body, such as homeostasis and regulation of the reproduction, development, metabolism and growth of individuals. The secretion of pituitary hormones is controlled by a positive feedback or a negative feedback mechanism involving hypothalamic hormone and the peripheral hormone secreted from the target endocrine gland. The

various receptor proteins present in the hypophysis are playing a central role in the regulation of the hypothalamus-pituitary system.

Meanwhile, it is known that these hormones and factors as well as their receptors are not localized in the hypothalamus-pituitary system but are broadly distributed in the brain. Therefore, it is suspected that, in the central nervous system, this substance called hypothalamus hormone is functioning as a neurotransmitter or a neuromodulator. Moreover, the substance is distributed in peripheral tissues as well and thought to be playing important roles in the respective tissue.

The pancreas is playing a crucial role in the carbohydrate metabolism by secreting glucagon and insulin as well as digestive juice. While insulin is secreted from the pancreatic  $\beta$  cells, its secretion is mainly stimulated by glucose. However, it is known that  $\beta$  cells have a variety of receptors and the secretion of insulin is controlled by a number of factors in addition to glucose as well as peptide hormones, e.g. galanine, somatostatin, gastric inhibitory polypeptide, glucagon, amylin, etc.; sugars, e.g. mannose etc.; amino acids, and neurotransmitters, among others.

The means only heretofore available for identifying ligands for said G protein-coupled receptor proteins is estimation from the homology in primary structure of G protein-coupled receptor proteins.

Recently, investigation for novel opioid peptides by introducing a cDNA coding for a receptor protein which a ligand is unknown, i.e. an orphan G protein-coupled receptor protein, into animal cells have been reported (Reinsheid, R. K. et al., Science, 270, 792-794, 1995, Menular, J.-C., et al., Nature 377, 532-535, 1995). However, in view of similarities to known G



protein-coupled receptor proteins and tissue distributions, it could be easily anticipated in these cases that the ligand would be belonging to the family of opioid peptides. The history of research and development in the realm of substances acting on the living body through the opioid receptor dates back to many years ago and various antagonists and agonists had been developed. Therefore, among the compounds artificially synthesized, an agonist of the receptor was picked out and, using it as a probe, expression of the receptor in the receptor cDNA-transfected cells was verified. Then, a search was made for an activator of the intracellular signal transduction which was similar to the agonist, the activator so found was purified, and the structure of the ligand was determined. However, when the homology of an orphan receptor to known G protein-coupled receptor proteins is low, it was very difficult to predict its ligand.

Ligands for orphan G protein-coupled receptors expressed in the hypophysis, central nervous system, and pancreatic  $\beta$  cells are considered to be useful for developing medicines, but their structures and functions have not been elucidated as yet.

[Disclosure of Invention]

Employing a cell in which a cDNA coding for orphan G protein-coupled receptor protein has been expressed by a suitable means and using measurement of a specific cell stimulation activity exemplified by a signal transduction activity as an indicator, the inventors of the present invention succeeded in screening a polypeptide which said receptor protein recognizes as a ligand.

Furthermore, the inventors found that a compound can be screened which is capable of changing the binding activity of this ligand which is an activating

factor to said receptor protein.

The present invention, therefore, relates to

(1) A polypeptide which comprises an amino acid sequence represented by SEQ ID NO:73 or its substantial equivalent thereto, or its amide or ester, or a salt thereof.

(2) The polypeptide as described in (1) above, which comprises the amino acid sequence represented by SEQ ID NO:3, SEC ID NO:4, SEQ ID NO:5, SEQ ID NO:6, SEQ ID NO:7, SEQ ID NO:8, SEQ ID NO:9, SEQ ID NO:10, SEQ ID NO:47, SEQ ID NO:48, SEQ ID NO:49, SEQ ID NO:50, SEQ ID NO:51, SEQ ID NO:52, SEQ ID NO:61, SEQ ID NO:62, SEQ ID NO:63, SEQ ID NO:64, SEQ ID NO:65, or SEQ ID NO:66.

(3) The polypeptide as described in (1) above, which comprises the amino acid sequence represented by SEQ ID NO:1, SEQ ID NO:44, SEQ ID NO:45, or SEQ ID NO:59.

(4) A partial peptide of the polypeptide as described in (1) above its amide or ester, or a salt thereof.

(5) A DNA which comprises a DNA having a nucleotide sequence coding for the polypeptide as described in (1) above or the partial peptide as described in (4) above.

(6) The DNA as described in (5) above which comprises a nucleotide sequence represented by SEQ ID NO:2, SEQ ID NO:11, SEQ ID NO:12, SEQ ID NO:13, SEQ ID NO:14, SEQ ID NO:15, SEQ ID NO:16, SEQ ID NO:17, SEQ ID NO:18, SEQ ID NO:18, SEQ ID NO:46, SEQ ID NO:53, SEQ ID NO:54, SEQ ID NO:55, SEQ ID NO:56, SEQ ID NO:57, SEQ ID NO:58, SEQ ID NO:60, SEQ ID NO:67, SEQ ID NO:68, SEQ ID NO:69, SEQ ID NO:70, SEQ ID NO:71, or SEQ ID NO:72.

(7) A recombinant vector comprising the DNA as described in (5) above.

(8) A transformant carrying the DNA as described in (5) above or the recombinant vector as described (7) above.

(9) A method for producing the polypeptide as described in (1) above or the partial peptide as

described in (4) above, which comprises culturing the transformant as described in (8) above.

(10) A pharmaceutical composition containing the polypeptide, its amide or ester as described in (1) above, or a pharmaceutically acceptable salt thereof.

(11) A pharmaceutical composition containing the partial peptide peptide, its amide or ester as described in (4) above, or a pharmaceutically acceptable salt thereof.

(12) A pharmaceutical composition containing the DNA as described in (5) above.

(13) The pharmaceutical composition as described in (10), (11), or (12) above, which is a pituitary function modulator.

(14) The pharmaceutical composition as described in (10), (11), or (12) above, which is a central nervous system function modulator.

(15) The pharmaceutical composition as described in (10), (11), or (12) above, which is a pancreatic function modulator.

(16) An antibody against the polypeptide as described in (1) above or against the partial peptide as described in (4) above.

(17) A screening method for a compound capable of changing the binding activity of the polypeptide as described in (1) above or the partial peptide as described in (4) above, with a receptor protein comprising an amino acid sequence represented by SEQ ID NO:21 or its partial peptide or its substantial equivalent thereto, or a salt thereof, which comprises making a comparison between: (i) at least one case where said polypeptide as described in (1) above or the partial peptide as described in (4) above is contacted with a receptor protein comprising an amino acid sequence represented by SEQ ID:21 or its partial peptide or its substantial equivalent thereto, or a

salt thereof, and (ii) at least one case where said polypeptide as described in (1) above or the partial peptide as described in (4) above together with a sample to be tested in contacted with protein comprising an amino acid sequence represented by SEQ ID NO:21 or its partial peptide or its substantial equivalent thereto, or a salt thereof.

(18) A kit for screening for a compound capable of changing the binding activity of the polypeptide as described in (1) above or the partial peptide as described in (4) above with a receptor protein comprising an amino acid sequence represented by SEQ ID NO:21 or its partial peptide or its substantial equivalent thereto, or a salt thereof.

(19) A compound capable of changing the binding activity of the polypeptide as described in (1) or the partial peptide as described in (4) with a receptor protein comprising an amino acid sequence represented by SEQ ID NO:21 or its partial peptide or its substantial equivalent thereto, or a salt thereof.

(20) A G protein-coupled receptor protein which recognizes the polypeptide as described in (1) above or the partial peptide as described in (4) above as a ligand, or a salt thereof.

The present invention further provides:

(21) the polypeptide as described in (1) above, or its amide or ester, or a salt thereof, which comprises an amino acid sequence selected from the group consisting of an amino acid sequence of SEQ ID NO:73, amino acid sequences wherein 1 to 15 amino acid residues, preferably 1 to 10 amino acid residues, more preferably 1 to 5 amino acid residues are deleted from the amino acid sequence of SEQ ID NO:73, amino acid sequences wherein 1 to 80 amino acid residues, preferably 1 to 50 amino acid residues, more preferably 1 to 10 amino acid residues are added to the amino acid sequence of SEQ ID

NO:73, and amino acid sequences wherein 1 to 15 amino acid residues, preferably 1 to 10 amino acid residues, more preferably 1 to 5 amino acid residues in the amino acid sequence of SEQ ID NO:73 are substituted with one or more other amino acid residues;

(22) the polypeptide as described in (1) above, which comprises an amino acid sequence wherein the peptide of SEQ ID NO:74 is added to the N-terminus of the polypeptide comprising the amino acid sequence of SEQ ID NO:73;

(23) the polypeptide as described in (1) above, which is derived from bovine, rat or human; and

(24) the pharmaceutical composition described in (10), (11) or (12) above, which is a therapeutic and/or prophylactic agent for dementia, depression (melancholia), hyperkinetic (microencephalo-pathy) syndrome, disturbance of consciousness, anxiety syndrome, schizophrenia, horror, growth hormone secretory disease, hyperphagia, polyphagia, hypercholesterolemia, hyperglyceridemia, hyperlipemia, hyperprolactinemia, diabetes, cancer, pancreatitis, renal disease, Turner's syndrome, neurosis, rheumatoid arthritis, spinal injury, transient brain ischemia, amyotrophic lateral sclerosis, acute myocardial infarction, spinocerebellar degeneration, bone fracture, trauma, atopic dermatitis, osteoporosis, asthma, epilepsy, infertility and/or oligogalactia.

Referring to the G protein-coupled receptor protein for the ligand polypeptide in accordance with the present invention, the invention specifically provides:

(25) the G protein-coupled receptor protein described in (20) or a salt thereof, which comprises an amino acid sequence represented by SEQ ID NO:19 or its substantial equivalent thereto or/and an amino acid sequence represented by SEQ ID NO:20 or its substantial

equivalene thereto;

(26) the G protein-coupled receptor protein described in (25) above or a salt thereof, which comprises an amino acid sequence represented by SEQ ID NO:21 or its substantial equivalent thereto;

(27) the G protein-coupled receptor protein described in (25) above or a salt thereof, which comprises an amino acid sequence represented by SEQ ID NO:22 or its substantial equivalent thereto;

(28) the G protein-coupled receptor protein described in (25) above or a salt thereof, which comprises an amino acid sequence represented by SEQ ID NO:23 or its substantial equivalent thereto;

(29) a partial peptide of any of the G protein-coupled receptor proteins described in (25)-(28) above or a salt thereof;

(30) a DNA which comprises a DNA having a nucleotide sequence coding for the G protein-coupled receptor protein described in (25) above;

(31) a DNA which comprises a DNA having a nucleotide sequence coding for the G protein-coupled receptor protein described in (26) above;

(32) a DNA which comprises a DNA having a nucleotide sequence coding for the G protein-coupled receptor protein described in (27) above;

(33) a DNA which comprises a DNA having a nucleotide sequence coding for the G protein-coupled receptor protein described in (28) above;

(34) the DNA described in (30) above, which comprises the nucleotide sequence of SEQ ID NO:24 or the nucleotide sequence of SEQ ID NO:25;

(35) the DNA described in (31) above, which comprises the nucleotide sequence of SEQ ID NO:26;

(36) the DNA described in (32) above, which comprises the nucleotide sequence of SEQ ID NO:27;

(37) the DNA described in (33) above, which comprises

the nucleotide sequence of SEQ ID NO:28;

(38) a recombinant vector comprising any of the DNAs described in (30)-(33) above;

(39) a transformant carrying the recombinant vector described in (38) above;

(40) a method for producing the G protein-coupled receptor protein described in (28) above or a salt thereof, which comprises culturing the transformant of (39) to produce said G protein-coupled receptor protein on the cell membrane of the transformant;

(41) an antibody to any of the G protein-coupled receptor protein described in (25)-(28) above or a salt thereof, or the partial peptide described in (29) above or a salt thereof.

To be further specific, the G protein-coupled receptor protein relates to:

(42) the G protein-coupled receptor protein described in (25) above or a salt thereof, wherein the protein comprises (i) an amino acid sequence selected from the group consisting of an amino acid sequence of SEQ ID NO:19, amino acid sequences wherein 1 to 30 amino acid residues, preferably 1 to 10 amino acid residues are deleted from the amino acid sequence of SEQ ID NO:19, amino acid sequences wherein 1 to 30 amino acid residues, preferably 1 to 10 amino acid residues are added to the amino acid sequence of SEQ ID NO:19, and amino acid sequences wherein 1 to 30 amino acid residues, preferably 1 to 10 amino acid residues in the amino acid sequence of SEQ ID NO:19 are substituted with one or more than amino acid residues and/or (ii) an amino acid sequence selected from the group consisting of an amino acid sequence of SEQ ID NO:20, amino acid sequences wherein 1 to 30 amino acid residues, preferably 1 to 10 amino acid residues are deleted from the amino acid sequence of SEQ ID NO:20, amino acid sequences wherein 1 to 30 amino acid

residues, preferably 1 to 10 amino acid residues are added to the amino acid sequence of SEQ ID NO:20, and amino acid sequences wherein 1 to 30 amino acid residues, preferably 1 to 10 amino acid residues in the amino acid sequence of SEQ ID NO:20 are substituted with one or more other amino acid residues;

(43) the G protein-coupled receptor protein described in (26) above or a salt thereof, wherein the protein comprises an amino acid sequence selected from the group consisting of an amino acid sequence of SEQ ID NO:21, amino acid sequences wherein 1 to 30 amino acid residues, preferably 1 to 10 amino acid residues are deleted from the amino acid sequence of SEQ ID NO:21, amino acid sequences wherein 1 to 30 amino acid residues, preferably 1 to 10 amino acid residues are added to the amino acid sequence of SEQ ID NO:21, and amino acid sequences wherein 1 to 30 amino acid residues, preferably 1 to 10 amino acid residues in the amino acid sequence of SEQ ID NO:21 are substituted with one or more other amino acid residues;

(44) the G protein-coupled receptor protein described in (27) above or a salt thereof wherein the protein comprises an amino acid sequence selected from the group consisting of an amino acid sequence of SEQ ID NO:22, amino acid sequences wherein 1 to 30 amino acid residues, preferably 1 to 10 amino acid residues are deleted from the amino acid sequence of SEQ ID NO:22, amino acid sequences wherein 1 to 30 amino acid residues, preferably 1 to 10 amino acid residues are added to the amino acid sequence of SEQ ID NO:22, and amino acid sequences wherein 1 to 30 amino acid residues, preferably 1 to 10 amino acid residues in the amino acid sequence of SEQ ID NO:22 are substituted with one or more other amino acid residues;

(45) the G protein-coupled receptor protein described in (28) above or a salt thereof, wherein the protein



comprises an amino acid sequence selected from the group consisting of an amino acid sequence of SEQ ID NO:23, amino acid sequences wherein 1 to 30 amino acid residues, preferably 1 to 10 amino acid residues are  
5 deleted from the amino acid sequence of SEQ ID NO:23, amino acid sequences wherein 1 to 30 amino acid residues, preferably 1 to 10 amino acid residues are added to the amino acid sequence of SEQ ID NO:23, and amino acid sequences wherein 1 to 30 amino acid  
10 residues, preferably 1 to 10 amino acid residues in the amino acid sequence of SEQ ID NO:23 are substituted with one or more other amino acid residues.

As used herein the term "substantial equivalent(s)" means that the activity of the protein,  
15 e.g., nature of the binding activity of the ligand and the receptor and physical characteristics are substantially the same. Substitutions, deletions or insertions of amino acids often do not produce radical changes in the physical and chemical characteristics of  
20 a polypeptide, in which case polypeptides containing the substitution, deletion, or insertion would be considered to be substantially equivalent to polypeptides lacking the substitution, deletion, or insertion. Substantially equivalent substitutes for an  
25 amino acid within the sequence may be selected from other members of the class to which the amino acid belongs. The non-polar (hydrophobic) amino acids include alanine, leucine, isoleucine, valine, proline, phenylalanine, tryptophan and methionine. The polar  
30 neutral amino acids include glycine, serine, threonine, cysteine, tyrosine, asparagine, and glutamine. The positively charged (basic) amino acids include arginine, lysine and histidine. The negatively charged (acidic) amino acids include aspartic acid and glutamic  
35 acid.

[Brief Description of the Drawings]

Fig. 1 shows the nucleotide sequence of the human pituitary-derived G protein-coupled receptor protein cDNA fragment harbored in cDNA clone p19P2 isolated by PCR using human pituitary-derived cDNA and the amino acid encoded by the nucleotide sequence. The primer used for sequencing was -21M13. The underscored region correspond to the synthetic primer.

Fig. 2 shows the nucleotide sequence of the human pituitary-derived G protein-coupled receptor protein cDNA fragment harbored in cDNA clone p19P2 isolated by PCR using human pituitary-derived cDNA and the amino acid sequence encoded thereby. The primer used for sequencing was M13RV-N (Takara). The underscored region correspond to the synthetic primer.

Fig. 3 shows a partial hydrophobic plot of the protein encoded by the human pituitary-derived G protein-coupled receptor protein cDNA fragment harbored in p19P2 constructed according to the amino acid sequence shown in Fig. 1.

Fig. 4 shows a partial hydrophobic plot of the protein encoded by the human pituitary-derived G protein-coupled receptor protein cDNA fragment harbored in p19P2 constructed according to the amino acid sequence shown in Fig. 2.

Fig. 5 is a diagram comparing the partial amino acid sequence of the protein encoded by the human pituitary-derived G protein-coupled receptor protein cDNA fragment harbored in p19P2 as shown in Figs. 1 and 2 with the known G protein-coupled receptor protein S12863. The shadowed region represents the region of agreement. The 1 to 9 amino acid sequence of p19P2 corresponds to the 1 to 99 amino acid sequence of Fig. 1 and the 156 to 230 amino acid sequence corresponds to the 1 to 68 amino acid sequence of Fig. 2.

Fig. 6 shows the nucleotide sequence of the MIN6-

derived G protein-coupled receptor protein cDNA  
 fragment based on the nucleotide sequences of the MIN6-  
 derived G protein-coupled receptor protein cDNA  
 fragments harbored in the cDNA clones pG3-2 and pG1-10  
 5 isolated by PCR using MIN6-derived cDNA and the amino  
 acid sequence encoded by the nucleotide sequence. The  
 underscored region correspond to the synthetic primer.

Fig. 7 is a diagram comparing the partial amino  
 acid sequence encoded by pG3-2/pG1-10 of the MIN6-  
 10 derived G protein-coupled receptor protein shown in  
 Fig. 6 with the partial amino acid sequence of the  
 protein encoded by p19P2 shown in Figs. 1 and 2. The  
 shadowed region corresponds to the region of agreement.  
 The 1 to 99 amino acid sequence of the protein encoded  
 15 by p19P2 corresponds to the 1 to 99 amino acid sequence  
 of Fig. 1 and the 156 to 223 amino acid sequence  
 corresponds to the 1 to 68 amino acid sequence of Fig.  
 2. The 1 to 223 amino acid sequence of the protein  
 encoded by pG3-2/pG1-10 corresponds to the 1 to 223  
 20 amino acid sequence of Fig. 6.

Fig. 8 is a partial hydrophobic plot of the MIN6-  
 derived G protein-coupled receptor protein constructed  
 according to the partial amino acid sequence shown in  
 Fig. 6.

Fig. 9 shows the entire nucleotide sequence of the  
 human pituitary-derived G protein-coupled receptor  
 protein cDNA harbored in the cDNA clone phGR3 isolated  
 from a human pituitary-derived cDNA library by the  
 plaque hybridization method using the DNA fragment  
 30 inserted in p19P2 as a probe and the amino acid  
 sequence encoded by the nucleotide sequence.

Fig. 10 shows the result of Northern blotting of  
 human pituitary mRNA hybridized with radioisotope-  
 labeled human pituitary cDNA clone phGR3.

Fig. 11 shows a hydrophobic plot of the protein  
 35 encoded by the human pituitary-derived G protein-

coupled receptor protein cDNA harbored in the phGR3 as constructed according to the amino acid sequence shown in Fig. 9.

Fig. 12 shows the nucleotide sequence of the MIN6-derived G protein-coupled receptor protein cDNA fragment harbored in the cDNA clone p5S38 isolated by PCR using MIN6-derived cDNA and the amino acid sequence encoded by the nucleotide sequence. The underscored region correspond to the synthetic primer.

Fig. 13 shows a diagram comparing the partial amino acid sequence of MIN6-derived G protein-coupled receptor protein encoded by p5S38 shown in Fig. 12 with the partial amino acid sequence of G protein-coupled receptor protein encoded by the cDNA fragment harbored in p19P2 as shown in Figs. 1 and 2 and the partial amino acid sequence of G protein-coupled receptor protein encoded by the nucleotide sequence generated from the nucleotide sequences of cDNA fragments contained in pG3-2 and pG1-10 shown in Fig. 6. The shadowed region represents the sequence region of agreement. The 1 to 144 amino acid sequence of the protein encoded by p5S38 corresponds to the 1 to 144 amino acid sequence of Fig. 12, the 1 to 99 amino acid sequence of the protein encoded by p19P2 corresponds to the 1 to 99 amino acid sequence of Fig. 1 and the 156 to 223 amino acid sequence corresponds to 1 to 68 amino acid sequence of Fig. 2. The 1 to 223 amino acid sequence of the protein encoded by pG3-2/pG1-10 corresponds to the 1 to 223 amino acid sequence of Fig. 6.

Fig. 14 shows a partial hydrophobic plot of the protein encoded by the MIN6-derived G protein-coupled receptor protein cDNA harbored in p5S38 as constructed according to the partial amino acid sequence shown in Fig. 12.

Fig. 15 shows the results of the following

analysis. Thus, RT-PCR was carried out to confirm the expression of mRNA in CHO cells transfected by pAKKO-19P2. Lanes 1-7 represent the results obtained by performing PCRs using serial dilutions of pAKKO-19P2 for comparison, i.e. the 10 $\mu$ l/ml stock solution (lane 1), 1/2 dilution (lane 2), 1/4 dilution (lane 3), 1/64 dilution (lane 4), 1/256 dilution (lane 5), 1/1024 dilution (lane 6), and 1/4096 dilution (lane 7) of the plasmid as templates, and analyzing the reaction mixtures by 1.2% agarose gel electrophoresis. Lanes 8 through 11 are the results obtained by performing PCRs using a 1/10 dilution (lane 8), a 1/100 dilution (lane 9), and a 1/1000 dilution (lane 10) of the cDNA prepared from the CHO-19P2 cell line as templates and subjecting the respective reaction mixtures to electrophoresis. Lane 11 was obtained by performing PCR using a template obtained by carrying out cDNA synthesis without reverse transcriptase and subjecting the PCR reaction product to electrophoresis. Lanes 12 and 13 were obtained by performing PCR using cDNAs prepared from mock CHO cells with and without addition of reverse transcriptase, respectively, as templates and subjecting the respective reaction products to electrophoresis. M represents the DNA size marker. The lanes at both ends were obtained by electrophoresing 1  $\mu$ l of  $\lambda$ /Sty I digest (Nippon Gene) and the second lane from right was obtained with 1  $\mu$ l of  $\phi$ / $\chi$ 174/Hinc II digest (Nippon Gene). The arrowmark indicates the position of the band amplified by PCR of about 400 bp.

Fig. 16 shows the activity of the crude ligand peptide fraction extracted from rat whole brain to promote release of arachidonic acid metabolites from CHO-19P2 cells. The arachidonic acid metabolite releasing activity was expressed as % of the amount of [ $^3$ H] arachidonic acid metabolites released in the

presence of the crude ligand polypeptide fraction with the amount of [ $^3\text{H}$ ] arachidonic acid metabolites released in the presence of 0.05% BAS-HABB being taken as 100%. The activity to promote release of

5 arachidonic acid metabolites from the CHO-19P2 cell line was detected in a 30%  $\text{CH}_3\text{CN}$  fraction.

Fig. 17 shows the activity of the crude ligand polypeptide fraction extracted from bovine hypothalamus to promote release of arachidonic acid metabolites from

10 CHO-19P2 cells. The arachidonic acid metabolite release-promoting activity was expressed as % of the amount of [ $^3\text{H}$ ] arachidonic acid metabolites released in the presence of the crude ligand polypeptide fraction with the amount of [ $^3\text{H}$ ] arachidonic acid metabolites

15 released in the presence of 0.05% BAS-HABB being taken as 100%. The activity to promote release of, arachidonic acid metabolites from the CHO-19P2 cell line was detected in a 30%  $\text{CH}_3\text{CN}$  fraction just as in the crude ligand polypeptide fraction from rat whole

20 brain.

Fig. 18 shows the activity of the fraction purified with the reversed-phase column C18 218TP5415 to specifically promote release of arachidonic acid metabolites from CHO-19P2 cells. The active fraction

25 from RESOURCE S was fractionated on C18 218TP5415. Thus, chromatography was carried out at a flow rate of 1 ml/min. on a concentration gradient of 20%-30%  $\text{CH}_3\text{CN}/0.1\%$  TFA/ $\text{H}_2\text{O}$ , the eluate was collected in 1 ml fractions, and each fraction was lyophilized. Then,

30 the activity of each fraction to specifically promote release of arachidonic acid metabolites from the CHO-19P2 cell line was determined. As a result, the activity was fractionated into 3 fractions (designated, in the order of elution, as P-1, P-2, and P-3).

35 Fig. 19 shows the activity of the fraction purified with the reversed-phase column diphenyl

219TP5415 to specifically promote arachidonic acid metabolite release from CHO-19P2 cells. The P-3 active fraction from C18 218TP5415 was fractionated on diphenyl 219TP5415. The chromatography was carried out at a flow rate of 1 ml/min. on a concentration gradient of 22%-25% CH<sub>3</sub>CN/0.1% TFA/H<sub>2</sub>O, the eluate was collected in 1 ml fractions, and each fraction was lyophilized. Then, the activity to specifically promote release of arachidonic acid metabolites from CHO-19P2 cells in each fraction was determined. As a result, the activity converged in a single peak.

Fig. 20 shows the activity of the fraction purified by reversed-phase column  $\mu$ RPC C2/C18 SC 2.1/10 to specifically promote release of arachidonic acid metabolites from CHO-19P2 cells. The peak active fraction from diphenyl 219TP5415 was fractionated on  $\mu$ RPC C2/C18 SC 2.1/10. The chromatography was carried out at a flow rate of 100  $\mu$ l/min. on a concentration gradient of 22%-23.5% CH<sub>3</sub>CN/0.1% TFA/H<sub>2</sub>O, the eluate was collected in 100  $\mu$ l fractions, and each fraction was lyophilized. Then, the activity to specifically promote release of arachidonic acid metabolites from CHO-19P2 cells in each fraction was determined. As a result, the activity was found as two peaks of apparently a single substance (peptide).

Fig. 21 shows the activity of the P-2 fraction purified by reversed-phase column  $\mu$ RPC C2/C18 SC 2.1/10 to specifically promote release of arachidonic acid metabolites from CHO-19P2 cells. The chromatography was carried out at a flow rate of 100  $\mu$ l/min. on a concentration gradient of 21.5%-23.0% CH<sub>3</sub>CN/0.1% TFA/dH<sub>2</sub>O, the eluate was collected in 100  $\mu$ l fractions, and each fraction was lyophilized. Then, the activity to specifically promote release of arachidonic acid metabolites from CHO-19P2 cells in each fraction was determined. As a result, the activity was found as a

peak of apparently a single substance.

Fig. 22 shows the nucleotide sequence of bovine hypothalamus ligand polypeptide cDNA fragment as derived from the nucleotide sequence of the bovine hypothalamus-derived ligand polypeptide cDNA fragment which specifically promotes release of arachidonic acid metabolites from CHO-19P2 cells as harbored in a cDNA clone isolated by PCR using bovine hypothalamus-derived cDNA and the amino acid sequence encoded by said nucleotide sequence. The region indicated by the arrowmark corresponds to the synthetic primer.

Fig. 23 shows the nucleotide sequence of the bovine hypothalamus-derived ligand polypeptide cDNA fragment generated according to the nucleotide sequence of the bovine hypothalamus-derived ligand polypeptide cDNA fragment which specifically promotes release of arachidonic acid metabolites from CHO-19P2 cells as harbored in a cDNA clone isolated by PCR using bovine hypothalamus-derived cDNA and the amino acid sequence encoded by said nucleotide sequence. The region indicated by the arrowmark corresponds to the synthetic primer.

Fig. 24 shows the amino acid sequences (a) and (b) of the bovine hypothalamus-derived ligand polypeptides which specifically promote release of arachidonic acid metabolites from CHO-19P2 cells and the cDNA sequence coding for the full coding region of the ligand polypeptides defined by SEQ ID NO:1 and SEQ ID NO:44.

Fig. 25 shows the concentration-dependent activity of synthetic ligand polypeptide (19P2-L31) to specifically promote release of arachidonic acid metabolites from CHO-19P2 cells. The synthetic peptide was dissolved in degassed  $\text{dH}_2\text{O}$  at a final concentration of  $10^{-3}\text{M}$  and diluted with 0.05% BSA-HBSS to concentrations of  $10^{-12}\text{M}$ - $10^{-6}\text{M}$ . The arachidonic acid metabolite releasing activity was expressed in the



measured radioactivity of [ $^3\text{H}$ ] arachidonic acid metabolites released in the supernatant when the dilution was added to the cells. As a result, the activity of 19P2-31 to specifically promote release of arachidonic acid metabolites from CHO-19P2 cells was found in a concentration-dependent manner.

Fig. 26 shows the concentration-dependent activity of synthetic ligand polypeptide (19P2-L31(O)) to specifically promote release of arachidonic acid metabolites from CHO-19P2 cells. The synthetic ligand peptide was dissolved in degassed  $\text{dH}_2\text{O}$  at a final concentration of  $10^{-3}\text{M}$  and diluted with 0.05% BSA-HBSS to concentrations of  $10^{-12}\text{M}$ - $10^{-6}\text{M}$ . The arachidonic acid metabolite releasing activity was expressed in the measured radioactivity of [ $^3\text{H}$ ] arachidonic acid metabolites released in the supernatant when the dilution was added to the cells. As a result, the activity of 19P2-L31(O) to specifically promote release of arachidonic acid metabolites from CHO-19P2 cells was found in a dose-dependent manner.

Fig. 27 shows the activity of synthetic ligand polypeptide 19P2-L20 to specifically promote release of arachidonic acid metabolites from CHO-19P2 cells. The synthetic peptide was dissolved in degassed  $\text{dH}_2\text{O}$  at a final concentration of  $10^{-3}\text{M}$  and diluted with 0.05% BSA-HBSS to concentrations of  $10^{-12}\text{M}$ - $10^{-6}\text{M}$ . The arachidonic acid metabolite releasing activity was expressed in the measured radioactivity of [ $^3\text{H}$ ] arachidonic acid metabolites released in the supernatant when the dilution was added to the cells. As a result, the activity of 19P2-L20 to specifically promote release of arachidonic acid metabolites from CHO-19P2 cells was found in a dose-dependent manner.

Fig. 28 shows the 1.2% agarose gel electrophoregram of the DNA fragments of the phages

cloned from a bovine genomic library as digested with restriction enzymes BamHI(B) and Sali(S). As the DNA size marker (M), StyI digests of  $\lambda$  phage DNA were used. In lane B, two bands derived from the vector were detected in positions between the first (19,329 bp) and second (7.743 bp) marker bands, as well as 3 bands derived from the inserted fragment between the third (6,223 bp) and 5th (3,472 bp) bands. In lane S, two bands derived from the vector were similarly detected but due to the overlap of the band of the inserted fragment, the upper band is thicker than the band in lane B.

Fig. 29 shows the nucleotide sequence around the coding region as decoded from bovine genomic DNA. The 1st to 3rd bases (ATG) correspond to the translation start codon and the 767th to 769th bases (TAA) correspond to the translation end codon.

Fig. 30 shows a comparison between the nucleotide sequence (genome) around the coding region as deduced from bovine genomic DNA and the nucleotide sequence (cDNA) of bovine cDNA cloned by PCR. The sequence region of agreement is indicated by shading. As to the 101st to 572nd region, there is no corresponding region in the nucleotide sequence of cDNA, indicating that it is an intron.

Fig. 31 shows the translation of the amino acid sequence encoded after elimination of the intron from the nucleotide sequence around the coding region as decoded from bovine genomic DNA.

Fig. 32 shows the full-length amino acid sequence and the cDNA sequence coding for the full coding region of rat ligand polypeptide.

Fig. 33 shows amino acid sequence of bovine ligand polypeptide and the nucleotide sequences of DNAs coding for bovine polypeptide and rat polypeptide. The arrowmark indicates the region corresponding to the

synthetic primer.

Fig. 34 shows the full-length amino acid sequence and the sequence of cDNA coding for the full coding region of human ligand polypeptide.

5 Fig. 35 shows a comparison of the amino acid sequences in the translation region of bovine ligand polypeptide, rat ligand polypeptide, and human ligand polypeptide.

10 Fig. 36 shows the results of receptor binding experiments on living cells wherein radioiodinated ligand polypeptide is used in the experiments.

Fig. 37 shows the results of measurements of release of arachidonic acid metabolites from CHO-19P2-9 and CHO-UHR1 by ligand polypeptide.

15 Fig. 38 shows the results of quantification of UHR-1 mRNA by RT-PCR in discrete regions of the brain and tissues in rats.

20 Fig. 39 shows the results of quantification of ligand polypeptide mRNA by RT-PCR in discrete regions of the brain and tissues in rats.

Fig. 40 shows effects of ligand polypeptide on glucose-induced increase in plasma insulin concentration, which is measured by radioimmunoassay.

25 Fig. 41 shows the results of measurements of motor activity by administration of 10 nmol of ligand polypeptide to mouse.

(a) relates to spontaneous motor activity and (b) relates to rearing.

30 Fig. 42 shows the results of measurements of motor activity by administration of 1 nmol of ligand polypeptide to mouse.

(a) relates to spontaneous motor activity and (b) relates to rearing.

35 Fig. 43 shows the results of measurements of motor activity by administration of 0.1 nmol of ligand polypeptide to mouse.

(a) relates to spontaneous motor activity and (b) relates to rearing.

Fig. 44 shows the results of measurements of motor activity by administration of 0.01 nmol of ligand polypeptide to mouse.

(a) relates to spontaneous motor activity and (b) relates to rearing.

Fig. 45 shows the results of measurements of body temperature which is measured at the time when the ligand polypeptide is administered to the lateral ventricle of mice. The administration of ligand polypeptide is carried out after 15 hours from administration of reserpine at a dose of 3 mg/kg, S.C.

In Fig. 45, the single star mark asterisk shows  $p < 0.05$  and the double star marks asterisks shows  $p < 0.01$ .

Fig. 46 illustrates the drawing in which the micro-injection cannula is inserted into the area postrema at an angle of  $20^\circ$ .

Fig. 47 shows the typical example of direct and average blood pressure which is measured after the injection of ligand polypeptide into the area postrema of rat. It is measured after the injection of 10 nmol of ligand polypeptide at the rate of 1  $\mu$ l/min, and under the condition of non-anesthesia.

Fig. 48 shows the results of measurements of growth hormone (GH) in plasma when 50 nmol of ligand polypeptide is administered into the third ventricle of rat after anesthesia by pentobarbital.

Fig. 49 shows the changes of secretion of GH in plasma by administration of 50 nmol of ligand polypeptide into the third ventricle in freely moving rats.

The ligand polypeptide or PBS was administered into the third ventricle. At 10 min later, 5  $\mu$ g/kg of GHRH was administered intravenously to the rat

conscious. GH levels were measured just prior to intraventricular administration (time 0) and 10, 20, 30, 40, and 60 min after the intravenous injection of GHRH.

5 In Fig. 49, the single star mark asterisk shows  $p < 0.05$  and the double star marks asterisks show  $p < 0.01$ .

Fig. 50 shows the relationship between the ligand polypeptide serum and the absorbance.

10 Fig. 51 shows the inhibition of the release of archidonic acid metabolites by anti-ligand polypeptide polyclonal antibody.

Fig. 52 shows the sequence of cDNA coding for UHR-1, which is constructed on pAKKO-UHR1-7.

15 [Best Mode for Carrying Out the Invention]

The ligand polypeptide according to the present invention is a polypeptide which is capable of binding to G protein-coupled receptor protein and comprising an amino acid sequence represented by SEQ ID NO:73 or its  
20 substantial equivalent thereto or a partial peptide thereof, or its amide or ester, or a salt thereof. In SEQ ID NO:73, Xaa at 10th position is Ala or Thr; Xaa at 11th position is Gly or Ser; and Xaa at 21th position is H, Gly, or GlyArg.

25 The above ligand polypeptide, its amide or ester, or a salt thereof (hereinafter sometimes referred to briefly as the ligand polypeptide or the polypeptide), processes for their production, and uses for the polypeptide are now described in detail.

30 The above ligand polypeptide of the present invention includes any polypeptides derived from any tissues, e.g. pituitary gland, pancreas, brain, kidney, liver, gonad, thyroid gland, gall bladder, bone marrow, adrenal gland, skin, muscle, lung, digestive canal,  
35 blood vessel, heart, etc.; or cells of man and other warm-blooded animals, e.g. guinea pig, rat, mouse,

swine, sheep, bovine, monkey, etc. and comprising an amino acid sequence represented by SEQ ID NO:73 or its substantial equivalent thereto. For example, in addition to the protein comprising the amino acid sequence of SEQ ID NO:73, the ligand polypeptide of the present invention includes the protein comprising an amino acid sequence having a homology of about 50-99.9%, preferably 70-99.9%, more preferably 80-99.9% and especially preferably 90-99.9% to the amino acid sequence of SEQ ID NO:73 and having qualitatively substantially equivalent activity to the protein comprising the amino acid sequence of SEQ ID NO:73. The term "substantially equivalent" means the nature of the receptor-binding activity, signal transduction activity and the like is equivalent. Thus, it is allowable that even differences among grades, such as the strength of receptor binding activity and the molecular weight of the polypeptide are present.

To be more specific, the ligand polypeptide of the present invention includes the polypeptide derived from the rat whole brain, bovine hypothalamus, or human whole brain and comprising the amino acid sequence of SEQ ID NO:73. In addition, the ligand polypeptide of the present invention includes the polypeptides which comprises substantial equivalent polypeptides such as polypeptides wherein 1 to 15, preferably 1 to 10, and more preferably 1 to 5 amino acid residues are deleted from the amino acid sequence of SEQ ID NO:73, polypeptides wherein 1 to 80, preferably 1 to 50, more preferably 1 to 10 amino acid residues are added to the amino acid sequence of SEQ ID NO:73, or polypeptides wherein 1 to 15, preferably 1 to 10, more preferably 1 to 5 amino acid residues are substituted with one or more other amino acid residues.

The amino acid sequence of SEQ ID NO:73 comprises SEQ ID NO:8, 9, 10, 50, 51, 52, 64, 65 or 66. The

substantial equivalent polypeptides to the polypeptide comprising the amino acid sequence of SEQ ID NO: 73 are polypeptides comprising the amino acid sequences of SEQ ID NO:1, 3, 4, 5, 6, 7, 44, 45, 47, 48, 49, 59, 61, 62, or 63.

Among them, preferred is the polypeptide comprising the amino acid sequence of SEQ ID NO:73 and the polypeptide comprising the amino acid sequence which a peptide of SEQ ID NO:74 is added to the N-terminus of the polypeptide comprising the amino acid sequence of SEQ ID NO:73.

Furthermore, the polypeptide or partial peptide of the present invention includes those wherein the N-terminal side of Gln is cleaved in vivo to form pyroglutamyl peptide.

The peptides described in this specification, the left ends are the N-terminus (amino terminus) and the right end is the C-terminus (carboxyl terminus) according to the convention of the peptide indication. While the C-terminus of the polypeptide of SEQ ID NO:73 is usually carboxyl ( $-\text{COOH}$ ) or carboxylate ( $-\text{COO}^-$ ), it may be amide ( $-\text{CONH}_2$ ) or ester ( $-\text{COOR}$ ) form. The ester residue R includes a  $\text{C}_{1-6}$  alkyl group such as methyl, ethyl, n-propyl, isopropyl, n-butyl, etc., a  $\text{C}_{3-8}$  cycloalkyl group such as cyclopentyl, cyclohexyl, etc., a  $\text{C}_{6-12}$  aryl group such as phenyl,  $\alpha$ -naphthyl, etc., and a  $\text{C}_{7-14}$  aralkyl group such as a phenyl- $\text{C}_{1-2}$  alkyl group, e.g. benzyl, phenethyl, benzhydryl, etc. or an  $\alpha$ -naphthyl- $\text{C}_{1-2}$  alkyl, e.g.  $\alpha$ -naphthylmethyl etc. In addition, the ester may be a pivaloyloxymethyl ester which is broadly used for oral administration. When the polypeptide of SEQ ID NO:73 has a carboxyl or carboxylate group in any position other than the C-terminus, the corresponding amide or ester are also included in the concept of the polypeptide of the present invention. The ester mentioned just above

includes the esters mentioned for the C-terminus.

The preferred ligand polypeptide of the present invention is a peptide which the C-terminus is amidated. Especially preferred is a polypeptide comprising the amino acid sequence of SEQ ID NO:5, 8, 47, 50, 61 or 64 which the C-terminus is amidated.

The salt of polypeptide of the present invention includes salts with physiologically acceptable bases, e.g. alkali metals or acids such as organic or inorganic acids, and is preferably a physiologically acceptable acid addition salt. Examples of such salts are salts thereof with inorganic acids, e.g. hydrochloric acid, phosphoric acid, hydrobromic acid or sulfuric acid, etc. and salts thereof with organic acids, e.g. acetic acid, formic acid, propionic acid, fumaric acid, maleic acid, succinic acid, tartaric acid, citric acid, malic acid, oxalic acid, benzoic acid, methanesulfonic acid or benzenesulfonic acid, etc.

The ligand polypeptide or amide or ester, or a salt thereof of the present invention may be manufactured from the tissues or cells of warm-blooded animals inclusive of human by purifying techniques or manufactured by the peptide synthesis as described hereinafter. Moreover, it can be manufactured by culturing a transformant carrying a DNA coding for the polypeptide as described hereinafter.

In the production from the tissues or cells of human or other warm-blooded animals, the ligand polypeptide can be purified and isolated by a process which comprises homogenizing the tissue or cells of human or other warm-blooded animal, extracting the homogenate with an acid, for instance, and subjecting the extract to a combination of chromatographic procedures such as reversed-phase chromatography, ion-exchange chromatography, affinity chromatography, etc.



As mentioned above, the ligand polypeptide in the present invention can be produced by the per se known procedures for peptide synthesis. The methods for peptide synthesis may be any of a solid-phase synthesis and a liquid-phase synthesis. Thus, the objective peptide can be produced by condensing a partial peptide or amino acid capable of constituting the protein with the residual part thereof and, when the product has a protective group, the protective group is detached whereupon a desired peptide can be manufactured. The known methods for condensation and deprotection includes the procedures described in the following literature (1)-(5).

- (1) M. Bodanszky and M. A. Ondetti, Peptide Synthesis, Interscience Publishers, New York, 1966
- (2) Schroeder and Luebke, The Peptide, Academic Press, New York, 1965
- (3) Nobuo Izumiya et al., Fundamentals and Experiments in Peptide Synthesis, Maruzen, 1975
- (4) Haruaki Yajima and Shumpei Sakakibara, Biochemical Experiment Series 1, Protein Chemistry IV, 205, 1977
- (5) Haruaki Yajima (ed.), Development of Drugs-Continued, 14, Peptide Synthesis, Hirokawa Shoten

After the reaction, the protein can be purified and isolated by a combination of conventional purification techniques such as solvent extraction, column chromatography, liquid chromatography, and recrystallization. Where the protein isolated as above is a free compound, it can be converted to a suitable salt by the known method. Conversely where the isolated product is a salt, it can be converted to the free peptide by the known method.

The amide of polypeptide can be obtained by using a resin for peptide synthesis which is suited for

amidation. The resin includes chloromethyl resin, hydroxymethyl resin, benzhydrylamine resin, aminomethyl resin, 4-benzyloxybenzyl alcohol resin, 4-methylbenzhydrylamine resin, PAM resin, 4-

5 hydroxymethylmethylphenylacetamidomethyl resin, polyacrylamide resin, 4-(2',4'-dimethoxyphenyl-hydroxymethyl)phenoxy resin, 4-(2',4'-dimethoxyphenyl-Fmoc aminoethyl)phenoxy resin, and so on. Using such a resin, amino acids whose  $\alpha$ -amino groups and functional

10 groups of side-chain have been suitably protected are condensed on the resin according to the sequence of the objective peptide by various condensation techniques which are known per se. At the end of the series of reactions, the peptide or the protected peptide is

15 removed from the resin and the protective groups are removed to obtain the objective polypeptide.

For the condensation of the above-mentioned protected amino acids, a variety of activating reagents for peptide synthesis can be used but a carbodiimide

20 compound is particularly suitable. The carbodiimide includes DCC, N,N'-diisopropylcarbodiimide, and N-ethyl-N'-(3-dimethylaminopropyl)carbodiimide. For activation with such a reagent, a racemization inhibitor additive, e.g. HOBT and the protected amino

25 acid are directly added to the resin or the protected amino acid pre-activated as symmetric acid anhydride, HOBT ester, or HOOBT ester is added to the resin. The solvent for the activation of protected amino acids or condensation with the resin can be properly selected

30 from among those solvents which are known to be useful for peptide condensation reactions. For example, N,N-dimethylformamide, N-methylpyrrolidone, chloroform, trifluoroethanol, dimethyl sulfoxide, DMF, pyridine, dioxane, methylene chloride, tetrahydrofuran,

35 acetonitrile, ethyl acetate, or suitable mixtures of them can be mentioned. The reaction temperature can be

selected from the range hitherto-known to be useful for peptide bond formation and is usually selected from the range of about  $-20^{\circ}\text{C}$  -  $50^{\circ}\text{C}$ . The activated amino acid derivative is generally used in a proportion of 1.5-4 fold excess. If the condensation is found to be insufficient by a test utilizing the ninhydrin reaction, the condensation reaction can be repeated to achieve a sufficient condensation without removing the protective group. If repeated condensation still fails to provide a sufficient degree of condensation, the unreacted amino group can be acetylated with acetic anhydride or acetylimidazole.

The protecting group of amino group for the starting material amino acid includes Z, Boc, tertiary-amyloxycarbonyl, isobornyloxycarbonyl, 4-methoxybenzyloxycarbonyl, Cl-Z, Br-Z, adamantyloxycarbonyl, trifluoroacetyl, phthalyl, formyl, 2-nitrophenylsulfenyl, diphenylphosphinothioyl, or Fmoc. The carboxy-protecting group that can be used includes but is not limited to the above-mentioned  $\text{C}_{1-6}$  alkyl,  $\text{C}_{3-8}$  cycloalkyl and  $\text{C}_{7-14}$  aralkyl as well as 2-adamantyl, 4-nitrobenzyl, 4-methoxybenzyl, 4-chlorobenzyl, phenacyl, benzyloxycarbonylhydrazido, tertiary-butoxycarbonylhydrazido, and tritylhydrazido.

The hydroxy group of serine and threonine can be protected by esterification or etherification. The group suited for said esterification includes carbon-derived groups such as lower alkanoyl groups, e.g. acetyl etc., aroyl groups, e.g. benzoyl etc., benzyloxycarbonyl, and ethoxycarbonyl. The group suited for said etherification includes benzyl, tetrahydropyranyl, and tertiary-butyl.

The protective group for the phenolic hydroxyl group of tyrosine includes Bzl,  $\text{Cl}_2$ -Bzl, 2-nitrobenzyl, Br-Z, and tertiary-butyl.

The protecting group of imidazole for histidine

includes Tos, 4-methoxy-2,3,6-trimethylbenzenesulfonyl, DNP, benzyloxymethyl, Bum, Boc, Trt, and Fmoc.

The activated carboxyl group of the starting amino acid includes the corresponding acid anhydride, azide, and active esters, e.g. esters with alcohols such as pentachlorophenol, 2,4,5-trichlorophenol, 2,4-dinitrophenol, cyanomethyl alcohol, p-nitrophenol, HONB, N-hydroxysuccinimide, N-hydroxyphthalimide, HOBT, etc. The activated amino group of the starting amino acid includes the corresponding phosphoramidate.

The method for elimination of protective groups includes catalytic reduction using hydrogen gas in the presence of a catalyst such as palladium black or palladium-on-carbon, acid treatment with anhydrous hydrogen fluoride, methanesulfonic acid, trifluoromethanesulfonic acid, trifluoroacetic acid, or a mixture of such acids, base treatment with diisopropylethylamine, triethylamine, piperidine, piperazine, reduction with sodium metal in liquid ammonia. The elimination reaction by the above-mentioned acid treatment is generally carried out at a temperature of  $-20^{\circ}\text{C}$  -  $40^{\circ}\text{C}$  and can be conducted advantageously with addition of a cation acceptor such as anisole, phenol, thioanisole, m-cresol, p-cresol, dimethyl sulfide, 1,4-butanedithiol, 1,2-ethanedithiol. The 2,4-dinitrophenyl group used for protecting the imidazole group of histidine can be eliminated by treatment with thiophenol, while the formyl group used for protecting the indole group of tryptophan can be eliminated by alkali treatment with dilute sodium hydroxide solution or dilute aqueous ammonia as well as the above-mentioned acid treatment in the presence of 1,2-ethanedithiol, 1,4-butanedithiol.

The method for protecting functional groups which should not take part in the reaction of the starting material, the protective groups that can be used, the

method of removing the protective groups, and the method of activating the functional groups that are to take part in the reaction can all be selected judiciously from among the known groups and methods.

5           An another method for obtaining the amide form of the polypeptide comprises amidating the  $\alpha$ -carboxyl group of the C-terminal amino acid at first, then extending the peptide chain to the N-side until the desired chain length, and then selectively deprotecting  
10 the  $\alpha$ -amino group of the C-terminal peptide and the  $\alpha$ -carboxy group of the amino acid or peptide that is to form the remainder of the objective polypeptide and condensing the two fragments whose  $\alpha$ -amino group and side-chain functional groups have been protected with  
15 suitable protective groups mentioned above in a mixed solvent such as that mentioned hereinbefore. The parameters of this condensation reaction can be the same as described hereinbefore. From the protected peptide obtained by condensation, all the protective  
20 groups are removed by the above-described method to thereby provide the desired crude peptide. This crude peptide can be purified by known purification procedures and the main fraction be lyophilized to provide the objective amidated polypeptide.

25           To obtain an ester of the polypeptide, the  $\alpha$ -carboxyl group of the C-terminal amino acid is condensed with a desired alcohol to give an amino acid ester and then, the procedure described above for production of the amide is followed.

30           The partial peptide of the ligand polypeptide of the present invention, its amide or ester, or a salt thereof can be any peptide that has the same activities, e.g. pituitary function modulating activity, central nervous system function modulating  
35 activity, or pancreatic function modulating activity as the polypeptide which has an amino acid sequence of SEQ

ID NO:73 or its substantial equivalent thereto. As such peptides, there can be mentioned peptides wherein 1 to 15 amino acids residues are deleted from the above-mentioned amino acid sequence of SEQ ID NO:73.

- 5 To be specific, the peptide having an amino acid sequence corresponding to the 2nd to 21st positions of the amino acid sequence of SEQ ID NO:73, the peptide corresponding to the 3rd to 21st positions of the amino acid sequence of SEQ ID NO:73, the peptide
- 10 corresponding to the 4th to 21st positions of the amino acid sequence of SEQ ID NO:73, the peptide corresponding to the 5th to 21st positions of the amino acid sequence of SEQ ID NO:73, the peptide corresponding to the 6th to 21st positions of the amino acid sequence of SEQ ID NO:73, the peptide
- 15 corresponding to the 7th to 21st positions of the amino acid sequence of SEQ ID NO:73, the peptide corresponding to the 8th to 21st positions of the amino acid sequence of SEQ ID NO:73, the peptide corresponding to the 9th to 21st positions of the amino acid sequence of SEQ ID NO:73, the peptide corresponding to the 10th to 21st positions of the amino acid sequence of SEQ ID NO:73, the peptide corresponding to the 11th to 21st positions of the amino acid sequence of SEQ ID NO:73,
- 20 the peptide corresponding to the 12th to 21st positions of the amino acid sequence of SEQ ID NO:73, the peptide corresponding to the 13th to 21st positions of the amino acid sequence of SEQ ID NO:73, the peptide corresponding to the 14th to 21st positions of the amino acid sequence of SEQ ID NO:73, and the peptide corresponding to the 15th to 21st positions of the amino acid sequence of SEQ ID NO:73, can be mentioned as preferred examples. Moreover, the peptide having the amino acid sequence of SEQ ID NO:74 is also
- 35 preferred.

The ligand polypeptide or partial peptide thereof

can be used as antigen for preparation of anti-ligand polypeptide antibody. The polypeptide as antigen includes N-terminus peptides, C-terminus peptides or peptides of central portions other than above-mentioned ligand polypeptides or partial peptides thereof. To be more specifically includes the partial peptide of SEQ ID NO: 92, 93 or 94.

The partial peptide may be a peptide containing each of the domains or a peptide containing a plurality of the domains within the molecule.

The partial peptide mentioned in this specification may be one ending with an amide bond ( $-\text{CONH}_2$ ) or an ester bond ( $-\text{COOR}$ ) at the C-terminus. The ester here includes the same one of the above polypeptide. When the partial peptide has a carboxyl or carboxylate group in any position other than the C-terminus, the case in which such group or moiety has been amidated or esterified also falls within the scope of the partial peptide in the present invention. The ester here may be of the same one as the above-mentioned ester at the C-terminus.

The ligand polypeptide or its partial peptide of the present invention may be in the form of a fused protein which fused with a protein whose functions or properties are already known.

The salt of such partial peptide of the ligand polypeptide of present invention may be of the same one as the above-mentioned salt of the polypeptide.

The partial peptide of the ligand polypeptide of the invention, its amide or ester, or a salt thereof can be produced by the same synthetic processes as mentioned for the polypeptide or by cleaving the polypeptide of the present invention with a suitable peptidase.

The DNA coding for the ligand polypeptide or a partial peptide thereof of the present invention may be

any DNA comprising the nucleotide sequence encoding a polypeptide having an amino acid sequence of SEQ ID NO:73 or its substantial equivalent thereto.

Furthermore, the DNA may be any of genomic DNA, genomic DNA library, tissue- or cell-derived cDNA, tissue- or cell-derived cDNA library, and synthetic DNA. The vector for such as library may be any of bacteriophage, plasmide, cosmide, and phagimide. Moreover, it can be directly amplified by the RT-PCR method by using an RNA fraction may be prepared from a tissue or cells .

To be more specific, as the DNA coding for a polypeptide derived from rat whole brain or bovine hypothalamus and comprising the amino acid sequence of SEQ ID NO:1 or SEQ ID NO:44, the DNA comprising the nucleotide sequence of SEQ ID NO:2 can be exemplified. In SEQ ID NO:2, R at 129th position represents G or A, and Y at 179th and 240th positions represents C or T. When Y at 179th position is C, the amino acid sequence of SEQ ID NO:1 is encoded, and when Y at 179th position is T, the amino acid sequence of SEQ ID NO:44 is encoded.

As the DNA coding for a bovine-derived polypeptide comprising the amino acid sequence of SEQ ID NO:3, 4, 5, 6, 7, 8, 9 or 10, a DNA comprising the nucleotide sequence of SEQ ID NO:11, 12, 13, 14, 15, 16, 17 or 18 can be exemplified. Here, R at 63th position of SEQ ID NO:11, 13, 14 or 15 and R at 29th position of SEQ ID NO:12, 16, 17, or 18 represent G or A.

As the DNA coding for a rat-derived polypeptide of SEQ ID NO:45, 47, 48, 49, 50, 51, or 52, a DNA comprising the nucleotide sequence of SEQ ID NO:46, 53, 54, 55, 56, 57, or 58 can be exemplified.

Furthermore, as the DNA coding for a human-derived peptide of SEQ ID NO:59, 61, 62, 63, 64, 65, or 66, a DNA comprising the nucleotide sequence of SEQ ID NO:60, 67, 68, 69, 70, 71, or 72 can be exemplified.



Among DNAs coding for the bovine-derived polypeptide comprising the amino acid sequence of SEQ ID NO:1 or SEQ ID NO:44, the rat-derived polypeptide comprising the amino acid sequence of SEQ ID NO:45, or the human-derived polypeptide comprising the amino acid sequence of SEQ ID NO:59, DNA fragments comprising partial nucleotide sequences of 6 to 90, preferably 6 to 60, more preferably 9 to 30, and especially preferably 12 to 30 can be advantageously used as DNA probes as well.

The DNA coding for the ligand polypeptide or a partial peptide thereof of the present invention can be produced by the following genetic engineering procedures.

The DNA fully encoding the polypeptide or partial peptide of the present invention can be cloned either by PCR amplification using synthetic DNA primers having a partial nucleotide sequence of the polypeptide or partial peptide or by hybridization using the DNA inserted in a suitable vector and labeled with a DNA fragment comprising a part or full region of a human-derived polypeptide or a synthetic DNA. The hybridization can be carried out typically by the procedure described in Molecular Cloning (2nd ed., J. Sambrook et al., Cold Spring Harbor Lab. Press, 1989). When a commercial library is used, the instructions given in the accompanying manual can be followed.

The cloned DNA coding for the polypeptide or partial peptide can be used directly or after digestion with a restriction enzyme or addition of a linker depending on purposes. This DNA has ATG as the translation initiation codon at the 5' end and may have TAA, TGA, or TAG as the termination codon at the 3' end. The translation initiation and termination codons can be added by means of suitable DNA adapters.

An expression vector for the polypeptide or

partial peptide can be produced by, for example (a) cutting out a target DNA fragment from the DNA for the polypeptide or partial peptide of the present invention and (b) ligating the target DNA fragment with the downstream side of a promoter in a suitable expression vector.

The vector may include plasmids derived from Escherichia coli, e.g., pBR322, pBR325, pUC12, pUC13, etc.; plasmids derived from Bacillus subtilis, e.g., pUB110, pTP5, pC194, etc.; plasmids derived from yeasts e.g., pSH19, pSH15, etc.; bacteriophages such as  $\lambda$  - phage, and animal virus such as retrovirus, vaccinia virus and baculovirus.

According to the present invention, any promoter can be used as long as it is compatible with the host cell which is used for expressing a gene. When the host for the transformation is E. coli, the promoters are preferably trp promoters, lac promoters, recA promoters,  $\lambda_{PL}$  promoters, lpp promoters, etc. When the host for the transformation is Bacillus, the promoters are preferably SPO1 promoters, SPO2 promoters, penP promoters, etc. When the host is a yeast, the promoters are preferably PHO5 promoters, PGK promoters, GAP promoters, ADH promoters, etc. When the host is an animal cell, the promoters include SV40-derived promoters, retrovirus promoters, metallothionein promoters, heat shock promoters, cytomegalovirus (CMV) promoters, SR $\alpha$  promoters, etc. An enhancer can be effectively utilized for expression.

As required, furthermore, a host-compatible signal sequence is added to the N-terminal side of the polypeptide or partial peptide thereof. When the host is E. coli, the utilizable signal sequences may include alkaline phosphatase signal sequences, OmpA signal sequences, etc. When the host is Bacillus, they may include  $\alpha$  -amylase signal sequences, subtilisin signal

sequences, etc. When the host is a yeast, they may include mating factor  $\alpha$  signal sequences, invertase signal sequences, etc. When the host is an animal cell, they may include insulin signal sequences,  $\alpha$ -interferon signal sequences, antibody molecule signal sequences, etc.

A transformant or transfectant is produced by using the vector thus constructed, which carries the polypeptide or partial peptide-encoding DNA of the present invention. The host may be, for example, Escherichia microorganisms, Bacillus microorganisms, yeasts, insect cells, animal cells, etc. Examples of the Escherichia and Bacillus microorganisms include Escherichia coli K12-DH1 [Proc. Natl. Acad. Sci. USA, Vol. 60, 160 (1968)], JM103 [Nucleic Acids Research, Vol. 9, 309 (1981)], JA221 [Journal of Molecular Biology, Vol. 120, 517 (1978)], HB101 [Journal of molecular Biology, Vol. 41, 459 (1969)], C600 [Genetics, Vol. 39, 440 (1954)], etc. Examples of the Bacillus microorganism are, for example Bacillus subtilis MI114 [Gene, Vol. 24, 255 (1983)], 207-21 [Journal of Biochemistry, Vol. 95, 76 (1984)], etc. The yeast may be, for example, Saccharomyces cerevisiae AH22, AH22R<sup>-</sup>, NA87-11A, DKD-5D, 20B-12, etc. The insect may include a silkworm (Bombyx mori larva), [Maeda et al, Nature, Vol. 315, 592 (1985)] etc. The host animal cell may be, for example, monkey-derived cell line, COS-7, Vero, Chinese hamster ovary cell line (CHO cell), DHFR gene-deficient Chinese hamster cell line (dhfr<sup>-</sup> CHO cell), mouse L cell, mouse myeloma cell, human FL, etc.

Depending on the host cell used, transformation is done using standard techniques appropriate to such cells. Transformation of Escherichia microorganisms can be carried out in accordance with methods as

disclosed in, for example, Proc. Natl. Acad. Sci. USA, Vol. 69, 2110 (1972), Gene, Vol. 17, 107 (1982), etc. Transformation of Bacillus microorganisms can be carried out in accordance with methods as disclosed in, for example, Molecular & General Genetics, Vol. 168, 111 (1979), etc. Transformation of the yeast can be carried out in accordance with methods as disclosed in, for example, Proc. Natl. Acad. Sci. USA, Vol. 75, 1929 (1978), etc. The insect cells can be transformed in accordance with methods as disclosed in, for example, Bio/Technology, 6, 47-55, 1988. The animal cells can be transformed by methods as disclosed in, for example, Virology, Vol. 52, 456, 1973, etc. The transformants or transfectants wherein the expression vector carrying a polypeptide or partial peptide thereof encoding DNA harbors are produced according to the aforementioned techniques.

Cultivation of the transformant (transfectant) in which the host is Escherichia or Bacillus microorganism can be carried out suitably in a liquid culture medium. The culture medium may contain carbon sources, nitrogen sources, minerals, etc. necessary for growing the transformant. The carbon source may include glucose, dextrin, soluble starch, sucrose, etc. The nitrogen source may include organic or inorganic substances such as ammonium salts, nitrates, corn steep liquor, peptone, casein, meat extracts, bean-cakes, potato extracts, etc. Examples of the minerals may include calcium chloride, sodium dihydrogen phosphate, magnesium chloride, etc. It is further allowable to add yeasts, vitamins, growth-promoting factors, etc. It is desired that the culture medium is pH from about 5 to about 8.

The Escherichia microorganism culture medium is preferably an M9 medium containing, for example, glucose and casamino acid (Miller, Journal of

Experiments in Molecular Genetics), 431-433, Cold Spring Harbor Laboratory, New York, 1972. Depending on necessity, the medium may be supplemented with drugs such as 3 $\beta$  -indolyl acrylic acid in order to improve efficiency of the promoter. In the case of an Escherichia host, the cultivation is carried out usually at about 15 to 43°C for about 3 to 24 hours. As required, aeration and stirring may be applied. In the case of Bacillus host, the cultivation is carried out usually at about 30 to 40°C for about 6 to 24 hours. As required, aeration and stirring may be also applied. In the case of the transformant in which the host is a yeast, the culture medium used may include, for example, a Burkholder minimum medium [Bostian, K.L. et al., Proc. Natl. Acad. Sci. USA, Vol. 77, 4505 (1980)], an SD medium containing 0.5% casamino acid [Bitter, G.A. et al., Proc. Natl. Acad. Sci. USA, Vol. 81, 5330 (1984)], etc. It is preferable that the pH of the culture medium is adjusted to be from about 5 to about 8. The cultivation is carried out usually at about 20 to 35°C for about 24 to 72 hours. As required, aeration and stirring may be applied. In the case of the transformant in which the host is an insect, the culture medium used may include those obtained by suitably adding additives such as passivated (or immobilized) 10% bovine serum and the like to the Grace's insect medium (Grace, T.C.C., Nature, 195, 788 (1962)). It is preferable that the pH of the culture medium is adjusted to be about 6.2 to 6.4. The cultivation is usually carried out at about 27°C for about 3 to 5 days. As desired, aeration and stirring may be applied. In the case of the transformant in which the host is an animal cell, the culture medium used may include MEM medium [Science, Vol. 122, 501 (1952)], DMEM medium [Virology, Vol. 8, 396 (1959)], RPMI 1640 medium [Journal of the American

Medical Association, Vol. 199, 519 (1967)], 199 medium [Proceedings of the Society of the Biological Medicine, Vol. 73, 1 (1950)], etc. which are containing, for example, about 5 to 20% of fetal calf serum. It is preferable that the pH is from about 6 to about 8. The cultivation is usually carried out at about 30 to 40°C for about 15 to 60 hours. As required, medium exchange, aeration and stirring may be applied.

Separation and purification of the polypeptide or partial peptide from the above-mentioned cultures can be carried out according to methods described herein below.

To extract polypeptide or partial peptide from the cultured microorganisms or cells, the microorganisms or cells are collected by known methods after the cultivation, suspended in a suitable buffer solution, disrupted by ultrasonic waves, lysozyme and/or freezing and thawing, etc. and, then, a crude extract of the polypeptide or partial peptide is obtained by centrifugation or filtration. Other conventional extracting or isolating methods can be applied. The buffer solution may contain a protein-denaturing agent such as urea or guanidine hydrochloride or a surfactant such as Triton X-100 (registered trademark, hereinafter often referred to as "TM").

In the case where the polypeptide or partial peptide are secreted into culture media, supernatant liquids are separated from the microorganisms or cells after the cultivation is finished and the resulting supernatant liquid is collected by widely known methods. The culture supernatant liquid and extract containing the polypeptide or partial peptide can be purified by suitable combinations of widely known methods for separation, isolation and purification. The widely known methods of separation, isolation and purification may include methods which utilizes

solubility, such as salting out or sedimentation with solvents methods which utilizes chiefly a difference in the molecular size or weight, such as dialysis, ultrafiltration, gel filtration and SDS-polyacrylamide gel electrophoresis, methods utilizing a difference in the electric charge, such as ion-exchange chromatography, methods utilizing specific affinity such as affinity chromatography, methods utilizing a difference in the hydrophobic property, such as reverse-phase high-performance liquid chromatography, and methods utilizing a difference in the isoelectric point such as isoelectric electrophoresis, or chromatofocusing, etc.

In cases where the polypeptide or partial peptide thus obtained is in a free form, the free protein can be converted into a salt thereof by known methods or method analogous thereto. In case where the polypeptide or partial peptide thus obtained is in a salt form vice versa, the protein salt can be converted into a free form or into any other salt thereof by known methods or method analogous thereto.

The polypeptide or partial peptide produced by the transformant can be arbitrarily modified or a polypeptide can be partly removed therefrom, by the action of a suitable protein-modifying enzyme before or after the purification. The protein-modifying enzyme may include trypsin, chymotrypsin, arginyl endopeptidase, protein kinase, glycosidase, etc. The activity of the polypeptide or partial peptide thus formed can be measured by experimenting the coupling (or binding) with receptor or by enzyme immunoassays (enzyme linked immunoassays) using specific antibodies.

The DNA coding for the ligand polypeptide of the present invention, the ligand polypeptide or a partial peptide thereof can be used for (1) synthesis of a part or the full length of the ligand for G protein-coupled

receptor protein, (2) search for the physiological activities of the ligand polypeptide or partial peptide thereof of the present invention, (3) preparation of a synthetic oligonucleotide probe or a PCR primer, (4) acquisition of DNAs coding for ligands of G protein-coupled receptor proteins and precursor proteins, (5) development of receptor-binding assay systems using the expression of recombinant receptor proteins and screening of candidate medicinally active compounds, (6) acquisition of antibodies and antisera, (7) development of diagnostic agents utilizing said antibodies or antisera, (8) development of drugs such as pituitary function modulators, central nervous system function modulators, and pancreatic function modulators, and (9) gene therapies, among other uses.

Particularly by using the receptor binding assay system using the expression of a recombinant G protein-coupled receptor protein, which is described hereinafter, agonists or antagonists of G protein-coupled receptors which are specific to warm-blood animals including humans can be screened and such agonists and antagonists can be used as prophylactic and therapeutic agents for various diseases.

Further, referring to (8) above, the ligand polypeptide, a partial peptide thereof, or the DNA encoding either of them of the present invention is useful as a safe pharmaceutical composition of low toxic potential because it is recognized as a ligand by the G protein-coupled receptor protein expressed in the hypophysis, central nervous system and pancreatic  $\beta$  cells. The ligand polypeptide, a partial peptide thereof, or the DNA encoding either of them of the present invention is associated with the modulation of pituitary function, central nervous system function, and pancreatic function and, therefore, can be used as a therapeutic and prophylactic pharmaceutical



composition for dementia such as senile dementia, cerebrovascular dementia (dementia due to cerebrovascular disorder), dementia associated with phylodegenerative retroplastic diseases (e.g.

5 Alzheimer's disease, Parkinson's disease, Pick's disease, Huntington's disease, etc.), dementia due to infectious diseases (e.g. delayed viral infections such as Creutzfeldt-Jakob disease), dementia associated with endocrine, metabolic, and toxic diseases (e.g.

10 hypothyroidism, vitamin B12 deficiency, alcoholism, and poisoning due to various drugs, metals, or organic compounds), dementia associated with oncogenous diseases (e.g. brain tumor), dementia due to traumatic diseases (e.g. chronic subdural hematoma):, depression

15 (melancholia), hyperkinetic (microencephalo-pathy) syndrome, disturbance of consciousness, anxiety syndrome, schizophrenia, horror, growth hormone secretory disease (e.g. gigantism, acromegalic gigantism etc.), hyperphagia, polyphagia,

20 hypercholesterolemia, hyperglyceridemia, hyperlipemia, hyperprolactinemia, diabetes (e.g. diabetic complications, diabetic nephropathy, diabetic neuropathy, diabetic retinopathy etc.), cancer (e.g. mammary cancer, lymphatic leukemia, cystic cancer,

25 ovary cancer, prostatic cancer etc.), pancreatitis, renal disease (e.g. chronic renal failure, nephritis etc.), Turner's syndrome, neurosis, rheumatoid arthritis, spinal injury, transient brain ischemia, amyotrophic lateral sclerosis, acute myocardial

30 infarction, spinocerebellar degeneration, bone fracture, trauma, atopic dermatitis, osteoporosis, asthma, epilepsy, infertility or oligogalactia. Furthermore, they can be also used as the agent for improvement in postoperative nutritional status and/or

35 vasopressor.

When the polypeptide, a partial peptide thereof,

or the DNA encoding either of them of the present invention is used as a pharmaceutical composition as described above, it can be used by conventional methods. For example, it can be used orally in the form of tablets which may be sugar coated as necessary, capsules, elixirs, microcapsules etc., or non-orally in the form of injectable preparations such as aseptic solutions and suspensions in water or other pharmaceutically acceptable liquids. These preparations can be produced by mixing the polypeptide, a partial peptide thereof, or the DNA encoding either of them with physiologically acceptable carriers, flavoring agents, excipients, vehicles, antiseptics, stabilizers, binders etc. in unit dosage forms required for generally accepted manners of pharmaceutical making. Active ingredient contents in these preparations are set so that an appropriate dose within the specified range is obtained.

Additives which can be mixed in tablets, capsules etc. include binders such as gelation, corn starch, tragacanth and gum arabic, excipients such as crystalline cellulose, swelling agents such as corn starch, gelatin and alginic acid, lubricants such as magnesium stearate, sweetening agents such as sucrose, lactose and saccharin, and flavoring agents such as peppermint, akamono oil and cherry. When the unit dosage form is the capsule, the above-mentioned materials may further incorporate liquid carriers such as oils and fats. Sterile compositions for injection can be formulated by ordinary methods of pharmaceutical making such as by dissolving or suspending active ingredients, naturally occurring vegetable oils such as sesame oil and coconut oil, etc. in vehicles such as water for injection.

Aqueous liquids for injection include physiological saline and isotonic solutions containing

glucose and other auxiliary agents, e.g., D-sorbitol, D-mannitol and sodium chloride, and may be used in combination with appropriate dissolution aids such as alcohols, e.g., ethanol, polyalcohols, e.g., propylene glycol and polyethylene glycol, nonionic surfactants, e.g., polysorbate 80 (TM) and HCO-50 etc. Oily liquids include sesame oil and soybean oil, and may be used in combination with dissolution aids such as benzyl benzoate and benzyl alcohol. Furthermore the above-mentioned materials may also be formulated with buffers, e.g., phosphate buffer and sodium acetate buffer; soothing agents, e.g., benzalkonium chloride, procaine hydrochloride; stabilizers, e.g., human serum albumin, polyethylene glycol; preservatives, e.g., benzyl alcohol, phenol; antioxidants etc. The thus-prepared injectable liquid is normally filled in an appropriate ampule. Because the thus-obtained preparation is safe and of low toxicity, it can be administered to humans or warm-blooded mammals, e.g., mouse, rats, guinea pig, rabbits, chicken, sheep, pigs, bovines, cats, dogs, monkeys, baboons, chimpanzees, for instance.

The dose of said polypeptide, a partial peptide thereof, or the DNA encoding either of them is normally about 0.1-100 mg, preferably 1.0-50 mg, and more preferably 1.0-20 mg per day for an adult (weighing 60 kg) in oral administration, depending on symptoms etc. In non-oral administration, it is advantageous to administer the polypeptide, a partial peptide thereof, or the DNA encoding either of them in the form of injectable preparation at a daily dose of about 0.01-30 mg, preferably about 0.1-20 mg, and more preferably about 0.1-10 mg per administration by an intravenous injection for an adult (weighing 60 kg), depending on subject of administration, target organ, symptoms, method of administration etc. For other animal

species, corresponding does as converted per 60 kg weight can be administered.

The G protein-coupled receptor protein for the above ligand polypeptide of the present invention may be any of G protein-coupled receptor proteins derived from various tissues, e.g. hypophysis, pancreas, brain, kidney, liver, gonad, thyroid gland, gall bladder, bone marrow, adrenal gland, skin, muscle, lung, alimentary canal, blood vessel, heart, etc. of human and other warm-blooded animals, e.g. guinea pig, rat, mouse, swine, sheep, bovine, monkey, etc.; and comprising an amino acid sequence of SEQ ID NO:19, 20, 21, 22 or 23, or substantial equivalent thereto. Thus, the G protein-coupled receptor protein of the present invention includes, in addition to proteins comprising the SEQ ID NO:19, 20, 21, 22 or 23, those proteins comprising amino acid sequences of about 90-99.9% homology to the amino acid sequence of SEQ ID NO:19, 20, 21, 22 or 23 and having qualitatively substantially equivalent activity to proteins comprising the amino acid sequence of SEQ ID NO:19, 20, 21, 22, or 23. The activities which these proteins are possessed may include ligand binding activity and signal transduction activity. The term "substantially equivalent" means that the nature of the ligand binding activity and the like is equivalent. Therefore, it is allowable that even differences among grades such as the strength of ligand binding activity and the molecular weight of receptor protein are present.

To be further specific, the G protein-coupled receptor proteins include human pituitary-derived G protein-coupled receptor proteins which comprises the amino acid sequence of SEQ ID NO:19 or/and SEQ ID NO:20, mouse pancreas-derived G protein-coupled receptor proteins which comprises the amino acid sequence of SEQ ID NO:22, and mouse pancreas-derived G

protein-coupled receptor proteins which comprises the amino acid sequence of SEQ ID NO:23. As the human pituitary-derived G protein-coupled receptor proteins which comprises the amino acid sequence of SEQ ID NO:19 and/or SEQ ID NO:20 include the human pituitary-derived G protein-coupled receptor protein which comprises the amino acid sequence of SEQ ID NO:21. The G protein-coupled receptor proteins further include proteins wherein 1 to 30 amino acid residues, preferably 1 to 10 amino acid residues are deleted from the amino acid sequence of SEQ ID NO:19, 20, 21, 22 or 23, proteins wherein 1 to 30 amino acid residues, preferably 1 to 10 amino acid residues are added to the amino acid sequence of SEQ ID NO:19, 20, 21, 22, or 23, the proteins wherein 1 to 30 amino acid residues, preferably 1 to 10 amino acid residues in the amino acid sequence of SEQ ID NO:19, 20, 21, 22, or 23 are substituted with one or more other amino acid residues.

Here, the protein which comprises an amino acid sequence of SEQ ID NO:21 or substantial equivalent thereto contains the full-length of the amino acid sequence for human pituitary-derived G protein-coupled receptor protein. The protein which comprises an amino acid sequence of SEQ ID NO:19 or/and SEQ ID NO:20 or substantial equivalent thereto may be a partial peptide of the protein which comprises an amino acid sequence of SEQ ID NO:21 or substantial equivalent thereto. The protein which comprises an amino acid sequence of SEQ ID NO:22 or SEQ ID NO:23 or substantial equivalent thereto is a G protein-coupled receptor protein which is derived from mouse pancreas but since its amino acid sequence is quite similar to the amino acid sequence of SEQ ID NO:19 or/and SEQ ID NO:20 (cf. Example 8, Fig. 13 in particular), the protein which comprises an amino acid sequence of SEQ ID NO:22 or 23 or substantial equivalent thereto is also subsumed in the category of

said partial peptide of the protein which comprises an amino acid sequence of SEQ ID NO:21 or substantial equivalent thereto.

Thus, the above-mentioned protein comprising an amino acid sequence of SEQ ID NO:21 or substantial equivalent thereto or a partial peptide of the protein or a salt thereof, which will be described below, includes the protein comprising an amino acid sequence of SEQ ID NO:19, 20, 22, or 23 or substantial equivalent thereto, or a salt thereof.

Furthermore, the G protein-coupled receptor protein includes the protein in which the N-terminal Met has been protected with a protective group, e.g. C<sub>1-6</sub> acyl such as formyl or acetyl, the protein in which the N-terminal side of Glu has been cleaved in vivo to form pyroglutamine, the protein in which the side chain of any relevant constituent amino acid has been protected with a suitable protective group, e.g. C<sub>1-6</sub> acyl such as formyl or acetyl, and the complex protein such as glycoproteins available upon attachment of sugar chains.

The salt of G protein-coupled receptor protein includes the same kinds of salts as mentioned for the ligand polypeptide.

The G protein-coupled receptor protein or a salt thereof or a partial peptide thereof can be produced from the tissues or cells of human or other warm-blooded animals by the per se known purification technology or, as described above, by culturing a transformant carrying a DNA coding for the G protein-coupled receptor protein. It can also be produced in accordance with the procedures for peptide synthesis which are described above.

A partial peptide of G protein-coupled receptor protein may include, for example, a fragment containing an extracellular portion of the G protein-coupled

receptor protein, i.e. the site which is exposed outside the cell membranes. Examples of the partial peptide are fragments containing a region which is an extracellular area (hydrophilic region) as analyzed in a hydrophobic plotting analysis of the G protein-coupled receptor protein, such as shown in Fig. 3, Fig. 4, Fig. 8, Fig. 11, or Fig. 14. Furthermore, a fragment which partly contains a hydrophobic region may also be used. While peptides which separately contains each domain may be used too, peptides which contains multiple domains at the same time will be used as well.

The salt of a partial peptide of G protein-coupled receptor protein may be the same one of salt mentioned for the salt of ligand polypeptide.

The DNA coding for the G protein-coupled receptor protein may be any DNA comprising a nucleotide sequence encoding the G protein-coupled receptor protein which comprises an amino acid sequence of SEQ ID NO:19, 20, 21, 22, or 23 or substantial equivalent thereto. It may also be any one of genomic DNA, genomic DNA library, tissue- or cell-derived cDNA, tissue- or cell-derived cDNA library, and synthetic DNA. The vector for such a library may include bacteriophage, plasmid, cosmid, and phagimide. Furthermore, using an RNA fraction prepared from a tissue or cells, a direct amplification can be carried out by the RT-PCR method.

To be specific, the DNA encoding the human pituitary-derived G protein-coupled receptor protein which comprises the amino acid sequence of SEQ ID NO:19 include a DNA which comprises the nucleotide sequence of SEQ ID NO:24. The DNA encoding the human pituitary-derived G protein-coupled receptor protein which comprises the amino acid sequence of SEQ ID NO:20 include a DNA which comprises the nucleotide sequence of SEQ ID NO:25. The DNA encoding the human pituitary-derived G protein-coupled receptor protein which

comprises the amino acid sequence of SEQ ID NO:21  
 include a DNA which comprises the nucleotide sequence  
 of SEQ ID NO:26. The DNA encoding the mouse pancreas-  
 derived G protein-coupled receptor protein which  
 5 comprises the amino acid sequence of SEQ ID NO:22  
 include a DNA which comprises the nucleotide sequence  
 of SEQ ID NO:27. The DNA encoding the mouse pancreas-  
 derived G protein-coupled receptor protein which  
 comprises the amino acid sequence of SEQ ID NO:23  
 10 include a DNA comprising the nucleotide sequence of SEQ  
 ID NO:28.

A method for cloning the DNA completely coding for  
 the G protein-coupled receptor protein, vector,  
 promoter, host cell, a method for transformation, a  
 15 method for culturing the transformant or a method for  
 separation and purification of the G protein-coupled  
 receptor protein may include the same one as mentioned  
 for the ligand polypeptide.

To be specific, the plasmid phGR3 obtained in  
 20 Example 5, described hereinafter, is digested with the  
 restriction enzyme SalI and the translation frame for  
 the full-length cDNA encoding hGR3 is isolated. This  
 frame is subjected to ligation to, for example, the  
 expression vector pAKKO-111 for animal cell use which  
 25 has been treated with BAP (bacterial alkaline  
 phosphatase) after SalI digestion for inhibition of  
 autocyclization. After completion of the ligation  
 reaction, a portion of the reaction mixture is used for  
 transfection of, for example, Escherichia coli DH5.  
 30 Among the transformants obtained, a transformant in  
 which the cDNA coding for hGR3 has been inserted in the  
 forward direction with respect to a promoter, such as  
 SR $\alpha$ , which has been inserted into the expression vector  
 beforehand is selected by mapping after cleavage with  
 35 restriction enzymes or by nucleotide sequencing and the  
 plasmid DNA is prepared on a production scale.



The thus-constructed DNA of the expression vector is introduced into CHO dhfr<sup>-</sup> cells using a kit for introducing a gene into animal cells by the calcium phosphate method, the liposome method or the like to provide a high G protein-coupled receptor protein (hGR3) expression CHO cell line.

The resulting CHO cells are cultured in a nucleic acid-free screening medium in a CO<sub>2</sub> incubator at 37°C using 5% CO<sub>2</sub> for 1-4 days so as to give the G protein-coupled receptor protein (hGR3).

The G protein-coupled receptor protein is purified from the above CHO cells using an affinity column prepared by conjugating an antibody to the G protein-coupled receptor protein or a partial peptide thereof to a support or an affinity column prepared by conjugating a ligand for the G protein-coupled receptor protein.

The activity of the G protein-coupled receptor protein thus formed can be measured by experimenting the binding with a ligand or by enzyme immunoassays using specific antibodies.

The G protein-coupled receptor protein, the partial peptide thereof and the G protein-coupled receptor protein-encoding DNA can be used for:

- 1) determining a ligand to the G protein-coupled receptor protein,
- 2) obtaining an antibody and an antiserum,
- 3) constructing a system for expressing a recombinant receptor protein,
- 4) developing a receptor-binding assay system using the above developing system and screening pharmaceutical candidate compounds,
- 5) designing drugs based upon comparison with ligands and receptors which have a similar or analogous structure,
- 6) preparing a probe for the analysis of genes and

preparing a PCR primer,

7) gene manipulation therapy,

In particular, it is possible to screen a G protein-coupled receptor agonist or antagonist specific to a warm-blooded animal such as human being by a receptor-binding assay system which uses a system for expressing a recombinant G protein-coupled receptor protein. The agonist or antagonist thus screened or characterized permits various applications including prevention and/or therapy of a variety of diseases.

Described below are uses of ligand polypeptide of the present invention, G protein-coupled receptor proteins to the ligand polypeptide, ligand polypeptide-encoding DNAs, G protein-coupled receptor protein-encoding DNAs and their antibodies.

(1) Method for Determining a Ligand to the G protein-coupled receptor Protein

The G protein-coupled receptor protein, the partial peptide thereof or a salt thereof is useful as a reagent for investigating or determining a ligand to said G protein-coupled receptor protein.

According to the present invention, methods for determining a ligand to the G protein-coupled receptor protein which comprises contacting the G protein-coupled receptor protein or the partial peptide thereof with the compound to be tested, and measuring the binding amount, the cell stimulating activity, etc. of the test compound to the G protein-coupled receptor protein or the partial peptide thereof are provided.

The compound to be tested may include not only known ligands such as angiotensins, bombesins, canavanoids, cholecystokinins, glutamine, serotonin, melatonins, neuropeptides Y, opioids, purine, vasopressins, oxytocins, VIP (vasoactive intestinal and related peptides), somatostatins, dopamine, motilins, amylin, bradykinins, CGRP (calcitonin gene related

peptides), leukotrienes, pancreastatins, prostaglandins, thromboxanes, adenosine, adrenaline,  $\alpha$ - and  $\beta$ - chemokines such as IL-8, GRO $\alpha$ , GRO $\beta$ , GRO $\gamma$ , NAP-2, ENA-78, PF4, IP10, GCP-2, MCP-1, HC14, MCP-3, I-309, 5 MIPl $\alpha$ , MIP-1 $\beta$ , RANTES, etc.; endothelins, enterogastrins, histamine, neurotensins, TRH, pancreatic polypeptides, galanin, modified derivatives thereof, analogues thereof, family members thereof and the like but also tissue extracts, cell culture 10 supernatants, etc. of human or warm-blooded animals such as mice, rats, swines, cattle, sheep and monkeys, etc. For example, said tissue extract, said cell culture supernatant, etc. is added to the G protein-coupled receptor protein for measurement of the cell 15 stimulating activity, etc. and fractionated by relying on the measurements whereupon a single ligand can be finally determined and obtained.

In one specific embodiment of the present invention, said method for determining the ligand 20 includes a method for determining whether a sample (including a compound or a salt thereof) is capable of stimulating a target cell which comprises binding said compound with the G protein-coupled receptor protein either in the presence of the G protein-coupled 25 receptor protein, the partial peptide thereof or a salt thereof, or in a receptor binding assay system in which the expression system for the recombinant receptor protein is constructed and used; and measuring the receptor-mediated cell stimulating activity, etc. 30 Examples of said cell stimulating activities that can be measured include promoting or inhibiting biological responses, e.g. liberation of arachidonic acid, liberation of acetylcholine, liberation of endocellular Ca<sup>2+</sup>, production of endocellular cAMP, production of 35 endocellular cGMP, production of inositol phosphate, changes in the cell membrane potential, phosphorylation

of endocellular protein, activation of c-fos, decrease in pH, etc, and preferably liberation of arachidonic acid. Examples of said compound or a salt thereof capable of stimulating the cell via binding with the G protein-coupled receptor protein include peptides, proteins, nonpeptidic compounds, synthetic compounds, fermented products, etc.

In more specific embodiments of the present invention, said methods for screening and identifying a ligand includes:

- 1) a method of screening for a ligand to a G protein-coupled receptor protein, which comprises contacting a labeled test compound with a G protein-coupled receptor protein or a salt thereof or its partial peptide or a salt thereof, and measuring the amount of the labeled test compound binding with said protein or salt thereof or with said partial peptide or salt thereof;
- 2) a method of screening for a ligand to a G protein-coupled receptor protein, which comprises contacting a labeled test compound with cells containing the G protein-coupled receptor protein or the membrane fraction of said cell, and measuring the amount of the labeled test compound binding with said cells or said membrane fraction;
- 3) a method of screening for a ligand to a G protein-coupled receptor protein, which comprises contacting a labeled test compound with the G protein-coupled receptor protein expressed on cell membranes by culturing transformants carrying the G protein-coupled receptor protein-encoding DNA and measuring the amount of the labeled test compound binding with said G protein-coupled receptor protein;
- 4) a method of screening for a ligand to a G protein-coupled receptor protein, which comprises contacting a test compound with cells containing the G protein-coupled receptor protein, and measuring the cell

stimulating activity, e.g. promoting or inhibiting activity on biological responses such as liberation of arachidonic acid, liberation of acetylcholine, liberation of endocellular  $\text{Ca}^{2+}$ , production of endocellular cAMP, production of endocellular cGMP, production of inositol phosphate, changes in the cell membrane potential, phosphorylation of endocellular protein, activation of c-fos, lowering in pH, etc. via the G protein-coupled receptor protein; and

5) a method of screening for a ligand to the G protein-coupled receptor protein, which comprises contacting a test compound with the G protein-coupled receptor protein expressed on the cell membrane by culturing transformants carrying the G protein-coupled receptor protein-encoding DNA, and measuring at least one cell stimulating activity, e.g., an activity for promoting or inhibiting physiological responses such as liberation of arachidonic acid, liberation of acetylcholine, liberation of endocellular  $\text{Ca}^{2+}$ , production of endocellular cAMP, production of endocellular cGMP, production of inositol phosphate, changes in the cell membrane potential, phosphorylation of endocellular protein, activation of c-fos, lowering in pH etc. via the G protein-coupled receptor protein.

Described below are specific illustrations of the method for screening and identifying ligands.

First, the G protein-coupled receptor protein used for the method for determining the ligand may include any material so far as it contains a G protein-coupled receptor protein, a partial peptide thereof or a salt thereof although it is preferable to express large amounts of the G protein-coupled receptor proteins in animal cells.

In the manufacture of the G protein-coupled receptor protein, the above-mentioned method can be used and carried out by expressing said protein

encoding DNA in mammalian cells or in insect cells. With respect to the DNA fragment coding for a particular region such as an extracellular epitope, the extracellular domains, etc., complementary DNA may be used although the method of expression is not limited thereto. For example, gene fragments or synthetic DNA may be used as well.

In order to introduce the G protein-coupled receptor protein-encoding DNA fragment into host animal cells and to express it efficiently, it is preferred that said DNA fragment is incorporated into the downstream side of polyhedron promoters derived from nuclear polyhedrosis virus belonging to baculovirus, promoters derived from SV40, promoters derived from retrovirus, metallothionein promoters, human heat shock promoters, cytomegalovirus promoters, SR $\alpha$  promoters, etc. Examinations of the quantity and the quality of the expressed receptor can be carried out by methods per se known to those of skill in the art or methods similar thereto based upon the present disclosure. For example, they may be conducted by methods described in publications such as Nambi, P. et al: The Journal of Biochemical Society, vol.267, pages 19555-19559 (1992).

Accordingly, with respect to the determination of the ligand, the material containing a G protein-coupled receptor protein or partial peptide thereof may include products containing G protein-coupled receptor proteins which are purified by methods per se known to those of skill in the art or methods similar thereto, peptide fragments of said G protein-coupled receptor protein, cells containing said G protein-coupled receptor protein, membrane fractions of the cell containing said protein, etc.

When the G protein-coupled receptor protein-containing cell is used in the determining method of the ligand, said cell may be immobilized with binding

agents including glutaraldehyde, formalin, etc. The immobilization may be carried out by methods per se known to those of skill in the art or methods similar thereto.

5           The G protein-coupled receptor protein-containing cells are host cells which express the G protein-coupled receptor protein. Examples of said host cells are microorganisms such as Escherichia coli, Bacillus subtilis, yeasts, insect cells, animal cells, etc.

10           The cell membrane fraction is a cell membrane-rich fraction which is prepared by methods per se known to those of skill in the art or methods similar thereto after disruption of cells. Examples of cell disruption may include a method for squeezing cells using a  
15   Potter-Elvehjem homogenizer, a disruption by a Waring blender or a Polytron manufactured by Kinematica, a disruption by ultrasonic waves, a disruption via blowing out cells from small nozzles together with applying a pressure using a French press or the like,  
20   etc. In the fractionation of the cell membrane, a fractionation method by means of centrifugal force such as a fractional centrifugal separation and a density gradient centrifugal separation is mainly used. For example, disrupted cellular liquid is centrifuged at a  
25   low speed (500 rpm to 3,000 rom) for a short period (usually, from about one to ten minutes), the supernatant liquid is further centrifuged at a high speed (15,000 rpm to 30,000 rom) usually for 30 minutes to two hours and the resulting precipitate is used as a  
30   membrane fraction. Said membrane fraction contains a lot of the expressed G protein-coupled receptor protein and a lot of membrane components such as phospholipids and membrane proteins derived from the cells.

          The amount of the G protein-coupled receptor  
35   protein in the membrane fraction cell containing said G protein-coupled receptor protein is preferably  $10^3$  to

10<sup>8</sup> molecules per cell or, more preferably, 10<sup>5</sup> to 10<sup>7</sup> molecules per cell. Incidentally, the greater the expressed amount, the higher the ligand binding activity (specific activity) per membrane fraction  
5 whereby the construction of a highly sensitive screening system becomes possible and, moreover, it permits measurement of a large amount of samples within the same lot.

In conducting the above-mentioned methods 1) to 3)  
10 wherein ligands capable of binding with the G protein-coupled receptor protein are determined, a suitable G protein-coupled receptor fraction and a labeled test compound are necessary. The G protein-coupled receptor fraction is preferably a naturally occurring (natural  
15 type) G protein-coupled receptor, a recombinant G protein-coupled receptor having the activity equivalent to that of the natural type. Here, the term "activity equivalent to" means the equivalent ligand binding activity, etc. as discussed above.

20 Suitable examples of the labeled test compound include above-mentioned compound to be tested which are labeled with [<sup>3</sup>H], [<sup>125</sup>I], [<sup>14</sup>C], [<sup>35</sup>S], etc.

Specifically, the determination of ligands capable of binding with G protein-coupled receptor proteins is  
25 carried out as follows:

First, cells or cell membrane fractions containing the G protein-coupled receptor protein are suspended in a buffer suitable for the assay to prepare the receptor sample for conducting the method of determining the  
30 ligand binding with the G protein-coupled receptor protein. The buffer may include any buffer such as Tris-HCL buffer or phosphate buffer with pH 4-10, preferably, pH 6-8, etc., as long as it does not inhibit the binding of the ligand with the receptor.  
35 In addition, surface-active agents such as CHAPS, Tween 80™ (Kao-Atlas, Japan), digitonin, deoxycholate, etc.



and various proteins such as bovine serum albumin (BSA), gelatin, milk derivatives, etc. may be added to the buffer with an object of decreasing the non-specific binding. Further, a protease inhibitor such as PMSF, leupeptin, E-64 (manufactured by Peptide Laboratory), pepstatin, etc. may be added with an object of inhibiting the decomposition of the receptor and the ligand by protease. A test compound labeled with a predetermined (or certain) amount (5,000 cpm to 500,000 cpm) of [ $^3\text{H}$ ], [ $^{125}\text{I}$ ], [ $^{14}\text{C}$ ], [ $^{35}\text{S}$ ], etc. coexists in 0.01 ml to 10 ml of said receptor solution. In order to know the non-specific binding amount (NSB), a reaction tube to which a great excessive amount of the unlabeled test compound is added is prepared as well. The reaction is carried out at 0-50°C, preferably at 4-37°C for 20 minutes to 24 hours, preferably 30 minutes to three hours. After the reaction, it is filtered through a glass fiber filter or the like, washed with a suitable amount of the same buffer and the radioactivity remaining in the glass fiber filter is measured by means of a liquid scintillation counter or a gamma-counter. The test compound in which the count (B - NSB) obtained by subtracting the non-specific binding amount (NSB) from the total binding amount (B) is more than 0 cpm is identified as a ligand to the G protein-coupled receptor protein.

In conducting the above-mentioned methods 4) to 5) wherein ligands capable of binding with the G protein-coupled receptor protein are determined, the cell stimulating activity, e.g. the liberation of arachidonic acid, the liberation of acetylcholine, endocellular  $\text{Ca}^{2+}$  liberation, endocellular cAMP production, the production of inositol phosphate, changes in the cell membrane potential, the phosphorylation of endocellular protein, the activation of c-fos, lowering of pH, the activation of G protein,

cell promulgation, etc.; mediated by the G protein-coupled receptor protein may be measured by known methods or by the use of commercially available measuring kits. To be more specific, G protein-coupled receptor protein-containing cells are at first cultured in a multi-well plate or the like.

In conducting the determination of ligand, it is substituted with a fresh medium or a suitable buffer which does not show toxicity to the cells in advance of the experiment, and incubated under appropriate conditions and for sufficient time after adding a test compound, etc. thereto. Then, the cells are extracted or the supernatant liquid is recovered and the resulting product is determined by each of the methods. When it is difficult to identify the production of the substance, e.g. arachidonic acid, etc. which is to be an index for the cell stimulating activity due to the decomposing enzyme contained in the cell, an assay may be carried out by adding an inhibitor against said decomposing enzyme. With respect to an activity such as an inhibitory action against cAMP production, it may be detected as an inhibitory action against the production of the cells whose fundamental production is increased by forskolin or the like.

The kit used for the method of determining the ligand binding with the G protein-coupled receptor protein includes a G protein-coupled receptor protein or a partial peptide thereof, cells containing the G protein-coupled receptor protein, a membrane fraction from the cells containing the G protein-coupled receptor protein, etc.

Examples of the kit for determining the ligand are as follows:

1. Reagent for Determining the Ligand.
  - 1) Buffer for Measurement and Buffer for Washing.
- The buffering product wherein 0.05% of bovine

serum albumin (manufactured by Sigma) is added to Hanks' Balanced Salt Solution (manufactured by Gibco).

This product may be sterilized by filtration through a membrane filter with a 0.45  $\mu$ m pore size, and stored at 4°C or may be formulated upon use.

2) G protein-coupled receptor Protein Sample.

CHO cells in which G protein-coupled receptor proteins are expressed are subcultured at the rate of  $5 \times 10^5$  cells/well in a 12-well plate and cultured at 37°C in a humidified 5% CO<sub>2</sub>/95% air atmosphere for two days to prepare the sample.

3) Labeled Test Compound.

The compound which is labeled with commercially available [<sup>3</sup>H], [<sup>125</sup>I], [<sup>14</sup>C], [<sup>35</sup>S], etc. or labeled with a suitable method.

The product in a state of an aqueous solution is stored at 4°C or at -20°C and, upon use, diluted to 1  $\mu$ M with a buffer for the measurement. In the case of a test compound which is barely soluble in water, it may be dissolved in an organic solvent such as dimethylformamide, DMSO, methanol and the like.

4) Unlabeled Test Compound.

The same compound as the labeled one is prepared in a concentration of 100 to 1,000-fold concentrated state.

2. Method of Measurement

1) G protein-coupled receptor protein-expressing CHO cells cultured in a 12-well tissue culture plate are washed twice with 1 ml of buffer for the measurement and then 490  $\mu$ l of buffer for the measurement is added to each well.

2) Five  $\mu$ l of the labeled test compound is added and the mixture is made to react at room temperature for one hour. For measuring the nonspecific binding amount, 5  $\mu$ l of the unlabeled test compound is added.

3) The reaction solution is removed from each well,

which is washed with 1 ml of a buffer for the measurement three times. The labeled test compound which is binding with the cells is dissolved in 0.2N NaOH-1% SDS and mixed with 4 ml of a liquid scintillator A manufactured by WAKO Pure Chemical, Japan.

4) Radioactivity is measured using a liquid scintillation counter such as one manufactured by Beckmann.

10 (2) Prophylactic and Therapeutic Agent for G protein-coupled receptor Protein or Ligand Polypeptide Deficiency Diseases

If a ligand to the G protein-coupled receptor protein is revealed via the aforementioned method (1), the ligand or the G protein-coupled receptor protein-encoding DNA can be used as a prophylactic and/or therapeutic agent for treating said G protein-coupled receptor protein or ligand polypeptide deficiency diseases depending upon the action that said ligand exerts.

For example, when there is a patient for whom the physiological action of the ligand, e.g. pituitary function modulating action, central nervous system function modulating action or pancreatic function modulating action; cannot be expected because of a decrease in the G protein-coupled receptor protein or ligand polypeptide in vivo, the amount of the G protein-coupled receptor protein or ligand polypeptide in the brain cells of said patient can be increased whereby the action of the ligand can be fully achieved by:

- (a) administering the G protein-coupled receptor protein-encoding DNA to the patient to express it; or
- (b) inserting the G protein-coupled receptor protein or ligand polypeptide-encoding DNA into brain cells or the like to said patient. Accordingly, the G protein-

coupled receptor protein- or ligand polypeptide-  
encoding DNA can be used as a safe and less toxic  
preventive and therapeutic agent for the G protein-  
coupled receptor protein or ligand polypeptide  
5 deficiency diseases.

When the above-mentioned DNA is used as the above-  
mentioned agent, said DNA may be used alone or after  
inserting it into a suitable vector such as retrovirus  
vector, adenovirus vector, adenovirus-associated virus  
10 vector, etc. followed by subjecting the product vector  
to a conventional means which is the same means as  
using the DNA coding for the ligand polypeptide or  
partial peptide thereof as the pharmaceutical  
composition.

15 (3) Quantitative Determination of the G protein-  
coupled receptor Protein to the Ligand Polypeptide  
The ligand polypeptide that has a binding property  
for a G protein-coupled receptor protein or a partial  
peptide thereof, or a salt thereof are capable of  
20 determining quantitatively an amount of a G protein-  
coupled receptor protein or a partial peptide thereof,  
or a salt thereof in vivo with good sensitivity.

This quantitative determination may be carried out  
by, for example, combining with a competitive analysis.  
25 Thus, a sample to be determined is contacted with the  
ligand polypeptide so that the concentration of a G  
protein-coupled receptor protein or a partial peptide  
thereof in said sample can be determined. In one  
embodiment of the quantitative determination, the  
30 protocols described in the following 1) and 2) or  
methods similar thereto may be used:

- 1) Hiroshi Irie (ed): "Radioimmunoassay" (Kodansha,  
Japan, 1974); and
  - 2) Hiroshi Irie (ed): "Radioimmunoassay, Second  
35 Series" (Kodansha, Japan, 1979).
- (4) Screening of Compound Changing the Binding

Activity of Ligand Polypeptide, Partial Peptide thereof or salt thereof (hereinafter sometimes referred to briefly as ligand or ligand polypeptide) with the G protein-coupled receptor Protein

5

G protein-coupled receptor proteins or partial peptide or salt thereof can be used. Alternatively, expression systems for recombinant G protein-coupled receptor proteins are constructed and receptor binding assay systems using said expression system are used. In these assay systems, it is possible to screen compounds, e.g. peptides, proteins, nonpeptidic compounds, synthetic compounds, fermented products, cell extracts, animal tissue extracts, etc.; or salts thereof which changes the binding activity of a ligand polypeptide with the G protein-coupled receptor protein. Such a compound includes a compound exhibiting a G protein-coupled receptor-mediated cell stimulating activity, e.g. activity of promoting or activity of inhibiting physiological reactions including liberation of arachidonic acid, liberation of acetylcholine, endocellular  $Ca^{2+}$  liberation, endocellular cAMP production, endocellular cGMP production, production of inositol phosphate, changes in cell membrane potential, phosphorylation of endocellular proteins, activation of c-fos, lowering of pH, activation of G protein, cell proliferation, etc.; so-called "G protein-coupled receptor-agonist", a compound free from such a cell stimulating activity, so-called "G protein coupled receptor-antagonist", etc. The term of "change the binding activity of a ligand polypeptide" includes the both concept of the case in which the binding of ligand is inhibited and the case in which the binding of ligand is promoted.

35

Thus, the present invention provides a method of screening for a compound which changes the binding

activity of a ligand with a G protein-coupled receptor protein or a salt thereof, characterized by comparing the following two cases:

- 5 (i) the case wherein the ligand is contacted with the G protein-coupled receptor protein or salt thereof, or a partial peptide thereof or a salt thereof; and
- (ii) the case wherein the ligand is contacted with a mixture of the G protein-coupled receptor protein or salt thereof or the partial peptide or salt thereof and  
10 said test compound.

In said screening method, one characteristic feature of the present invention resides in that the amount of the ligand bonded with said G protein-coupled receptor protein or the partial peptide thereof, the  
15 cell stimulating activity of the ligand, etc. are measured in both the case where (i) the ligand polypeptide is contacted with G protein-coupled receptor proteins or partial peptide thereof and in the case where (ii) the ligand polypeptide and the test  
20 compound are contacted with the G protein-coupled receptor protein or the partial peptide thereof, respectively and then compared therebetween.

In one more specific embodiment of the present invention, the following is provided:

- 25 1) a method of screening for a compound or a salt thereof which changes the binding activity of a ligand polypeptide with a G protein-coupled receptor protein, characterized in that, when a labeled ligand polypeptide is contacted with a G protein-coupled  
30 receptor protein or a partial peptide thereof and when a labeled ligand polypeptide and a test compound are contacted with a G protein-coupled receptor protein or a partial peptide thereof, the amounts of the labeled ligand polypeptide bonded with said protein or a  
35 partial peptide thereof or a salt thereof are measured and compared;

- 2) a method of screening for a compound or a salt thereof which changes the binding activity of a ligand polypeptide with a G protein-coupled receptor protein, characterized in that, when a labeled ligand polypeptide is contacted with cells containing G protein-coupled receptor proteins or a membrane fraction of said cells and when a labeled ligand polypeptide and a test compound are contacted with cells containing G protein-coupled receptor proteins or a membrane fraction of said cells, the amounts of the labeled ligand polypeptide binding with said protein or a partial peptide thereof or a salt thereof are measured and compared;
- 3) a method of screening for a compound or a salt thereof which changes the binding activity of a ligand polypeptide with a G protein-coupled receptor protein, characterized in that, when a labeled ligand polypeptide is contacted with G protein-coupled receptor proteins expressed on the cell membrane by culturing a transformant carrying a G protein-coupled receptor protein-encoding DNA and when a labeled ligand polypeptide and a test compound are contacted with G protein-coupled receptor proteins expressed on the cell membrane by culturing a transformant carrying a G protein-coupled receptor protein-encoding DNA, the amounts of the labeled ligand polypeptide binding with said G protein-coupled receptor protein are measured and compared;
- 4) a method of screening for a compound or a salt thereof which changes the binding of a ligand polypeptide with a G protein-coupled receptor protein, characterized in that, when a G protein-coupled receptor protein-activating compound, e.g. a ligand polypeptide of the present invention, etc. is contacted with cells containing G protein-coupled receptor proteins and when the G protein-coupled receptor



protein-activating compound and a test compound are contacted with cells containing G protein-coupled receptor proteins, the resulting G protein-coupled receptor protein-mediated cell stimulating activities, e.g. activities of promoting or activities of inhibiting physiological responses including liberation of arachidonic acid, liberation of acetylcholine, endocellular  $\text{Ca}^{2+}$  liberation, endocellular cAMP production, endocellular cGMP production, production of inositol phosphate, changes in cell membrane potential, phosphorylation of endocellular proteins, activation of c-fos, lowering of pH, activation of G protein, cell promulgation, etc.; are measured and compared; and

5) a method of screening for a compound or a salt thereof which changes the binding activity of a ligand polypeptide with a G protein-coupled receptor protein, characterized in that, when a G protein-coupled receptor protein-activating compound, e.g. a ligand polypeptide of the present invention, etc. is contacted with G protein-coupled receptor proteins expressed on cell membranes by culturing transformants carrying G protein-coupled receptor protein-encoding DNA and when a G protein-coupled receptor protein-activating compound and a test compound are contacted with the G protein-coupled receptor protein expressed on the cell membrane by culturing the transformant carrying the G protein-coupled receptor protein-encoding DNA, the resulting G protein-coupled receptor protein-mediated cell stimulating activities, e.g. activities of promoting or activities of inhibiting physiological responses such as liberation of arachidonic acid, liberation of acetylcholine, endocellular  $\text{Ca}^{2+}$  liberation, endocellular cAMP production, endocellular cGMP production, production of inositol phosphate, changes in cell membrane potential, phosphorylation of endocellular proteins, activation of c-fos, lowering of

pH, activation of G protein, and cell promulgation, etc.; are measured and compared.

5 The G protein-coupled receptor agonist or antagonist have to be screened by, first, obtaining a candidate compound by using G protein-coupled receptor protein-containing cells, tissues or cell membrane fractions derived from rat or the like (primary screening), then, making sure whether the candidate  
10 compound really inhibits the binding between human G protein-coupled receptor proteins and ligands (secondary screening). Other receptor proteins inevitably exist and when the cells, the tissues or the cell membrane fractions were used, they intrinsically make it difficult to screen agonists or antagonists to  
15 the desired receptor proteins. By using the human-derived G protein-coupled receptor protein, however, there is no need of effecting the primary screening, whereby it is possible to efficiently screen a compound that changes the binding activity between a ligand and  
20 a G protein-coupled receptor. Additionally, it is possible to evaluate whether the compound that is screened is a G protein-coupled receptor agonist or a G protein-coupled receptor antagonist.

25 Specific explanations of the screening method will be given as hereunder.

First, with respect to the G protein-coupled receptor protein used for the screening method of the present invention, any product may be used so far as it contains G protein-coupled receptor proteins or partial  
30 peptides thereof although the use of a membrane fraction of mammalian organs is preferable. However, human organs can be extremely scarce and, accordingly, G protein-coupled receptor proteins which are expressed in a large amount using a recombinant technique are  
35 suitable for the screening.

In the manufacture of the G protein-coupled

receptor protein, the above-mentioned method can be used.

When the G protein-coupled receptor protein-containing cells or cell membrane fractions are used in the screening method, the above-mentioned method can be used.

In conducting the above-mentioned methods 1) to 3) for screening the compound capable of changing the binding activity of the ligand with the G protein-coupled receptor protein, a suitable G protein-coupled receptor fraction and a labeled ligand polypeptide are necessary. With respect to the G protein-coupled receptor fraction, it is preferred to use naturally occurring G protein-coupled receptors (natural type G protein-coupled receptors) or recombinant type G protein-coupled receptor fractions with the activity equivalent to that of the natural type G protein coupled. Here the term "activity equivalent to" means the same ligand binding activity, or the substantially equivalent ligand binding activity.

With respect to the labeled ligand, it is possible to use labeled ligands, labeled ligand analogized compounds, etc. For example, ligands labeled with [ $^3\text{H}$ ], [ $^{125}\text{I}$ ], [ $^{14}\text{C}$ ], [ $^{35}\text{S}$ ], etc. and other labeled substances may be utilized.

Specifically, G protein-coupled receptor protein-containing cells or cell membrane fractions are first suspended in a buffer which is suitable for the determining method to prepare the receptor sample in conducting the screening for a compound which changes the binding activity of the ligand with the G protein-coupled receptor protein. With respect to the buffer, any buffer such as Tris-HCl buffer or phosphate buffer of pH 4-10, preferably, pH 6-8 which does not inhibit the binding of the ligand with the receptor may be used.

In addition, a surface-active agent such as CHAPS, Tween 80<sup>TM</sup> (Kao-Atlas, Japan), digitonin, deoxycholate, etc. and/or various proteins such as bovine serum albumin (BSA), gelatine, etc. may be added to the  
5 buffer with an object of decreasing the nonspecific binding. Further, a protease inhibitor such as PMSF, leupeptin, E-64 manufactured by Peptide Laboratory, Japan, pepstatin, etc. may be added with an object of inhibiting the decomposition of the receptor and the  
10 ligand by protease. A labeled ligand in a certain amount (5,000 cpm to 500,000 cpm) is added to 0.01 ml to 10 ml of said receptor solution and, at the same time,  $10^{-4}$ M to  $10^{-10}$ M of a test compound coexists. In order to determine the nonspecific binding amount  
15 (NSB), a reaction tube to which a great excessive amount of unlabeled test compounds is added is prepared as well.

The reaction is carried out at 0-50°C, preferably at 4-37°C for 20 minutes to 24 hours, preferably 30  
20 minutes to three hours. After the reaction, it is filtered through a glass fiber filter, a filter paper, or the like, washed with a suitable amount of the same buffer and the radioactivity retained in the glass fiber filter, etc. is measured by means of a liquid  
25 scintillation counter of a gamma-counter. Supposing that the count ( $B_0 - \text{NSB}$ ) obtained by subtracting the nonspecific binding amount (NSB) from the total binding amount ( $B_0$ ) wherein an antagonizing substance is not present is set at 100%, a test compound in which the  
30 specific binding amount ( $B - \text{NSB}$ ) obtained by subtracting the nonspecific binding amount (NSB) from the total binding amount (B) is, for example, less than 50% may be selected as a candidate ligand to the G protein-coupled receptor protein of the present  
35 invention.

In conducting the above-mentioned methods 4) to 5)

for screening the compound which changes the binding activity of the ligand with the G protein-coupled receptor protein, the G protein-coupled receptor protein-mediated cell stimulating activity, e.g. activities of promoting or activities of inhibiting physiological responses such as release of arachidonic acid, release of acetylcholine, intracellular  $\text{Ca}^{2+}$  increase, intracellular cAMP production, production of inositol phosphate, changes in the cell membrane potential, phosphorylation of intracellular proteins, activation of c-fos, lowering of pH, activation of G protein and cell proliferation, etc.; may be measured by known methods or by the use of commercially available measuring kits. To be more specific, G protein-coupled receptor protein-containing cells are at first cultured in a multiwell plate or the like.

In conducting the screening, it is substituted with a suitable buffer which does not show toxicity to fresh media or cells in advance, incubated under appropriate conditions and for a specified time after adding a test compound, etc. thereto. The resultant cells are extracted or the supernatant liquid is recovered and the resulting product is determined, preferably quantitatively, by each of the methods. When it is difficult to identify the production of the indicative substance, e.g. arachidonic acid, etc. which is to be an indication for the cell stimulating activity due to the presence of decomposing enzymes contained in the cell, an assay may be carried out by adding an inhibitor against said decomposing enzyme. With respect to the activities such as an inhibitory action against cAMP production, it may be detected as an inhibitory action against the cAMP production in the cells whose fundamental production has been increased by forskolin or the like.

In conducting a screening by measuring the cell

stimulating activity, cells in which a suitable G protein-coupled receptor protein is expressed are necessary. Preferred G protein-coupled receptor protein-expressing cells are naturally occurring G protein-coupled receptor protein (natural type G protein-coupled receptor protein)-containing cell lines or strains, e.g. mouse pancreatic  $\beta$  cell line, MIN6, etc., the above-mentioned recombinant type G protein-coupled receptor protein-expressing cell lines or strains, etc.

Examples of the test compound includes peptide, proteins, non-peptidic compounds, synthesized compounds, fermented products, cell extracts, plant extracts, animal tissue extracts, serum, blood, body fluid, etc. Those compounds may be novel or known.

A kit for screening the compound which changes the binding activity of the ligand with the G protein-coupled receptor protein or a salt thereof comprises a G protein-coupled receptor protein or a partial peptide thereof, or G protein-coupled receptor protein-containing cells or cell membrane fraction thereof.

Examples of the screening kit include as follows:

1. Reagent for Determining Ligand.

1) Buffer for Measurement and Buffer for Washing.

The product wherein 0.05% of bovine serum albumin (manufactured by Sigma) is added to Hanks' Balanced Salt Solution (manufactured by Gibco).

This may be sterilized by filtration through a membrane filter with a 0.45  $\mu$ m pore size, and stored at 4°C or may be prepared upon use.

2) Sample of G protein-coupled receptor Protein.

CHO cells in which a G protein-coupled receptor protein is expressed are subcultured at the rate of  $5 \times 10^5$  cells/well in a 12-well plate and cultured at 37°C with a 5% CO<sub>2</sub> and 95% air atmosphere for two days to prepare the sample.

### 3) Labeled Ligand.

The ligand which is labeled with commercially available [ $^3\text{H}$ ], [ $^{125}\text{I}$ ], [ $^{14}\text{C}$ ], [ $^{35}\text{S}$ ], etc.

The product in a state of an aqueous solution is stored at 4°C or at -20°C and, upon use, diluted to 1  $\mu\text{M}$  with a buffer for the measurement.

### 4) Standard Ligand Solution.

Ligand is dissolved in PBS containing 0.1% of bovine serum albumin (manufactured by Sigma) to make 1 mM and stored at -20°C.

## 2. Method of the Measurement.

1) CHO cells are cultured in a 12-well tissue culture plate to express G protein-coupled receptor proteins. The G protein-coupled receptor protein-expressing CHO cells are washed with 1 ml of buffer for the measurement twice. Then 490  $\mu\text{l}$  of buffer for the measurement is added to each well.

2) Five  $\mu\text{l}$  of a test compound solution of  $10^{-3}$  to  $10^{-10}$  M is added, then 5  $\mu\text{l}$  of a labeled ligand is added and is made to react at room temperature for one hour. For knowing the non-specific binding amount, 5  $\mu\text{l}$  of the ligand of  $10^{-3}$  M is added instead of the test compound.

3) The reaction solution is removed from the well, which is washed with 1 ml of buffer for the measurement three times. The labeled ligand binding with the cells is dissolved in 0.2N NaOH-1% SDS and mixed with 4 ml of a liquid scintillator A (such as manufactured by Wako Pure Chemical, Japan).

4) Radioactivity is measured using a liquid scintillation counter (e.g., one manufactured by Beckmann) and PMB (percent maximum binding) is calculated by the following equation:

$$\text{PMB} = [(B - \text{NSB}) / (B_0 - \text{NSB})] \times 100$$

PMB: Percent maximum binding

B: Value when a sample is added

NSB: Nonspecific binding

$B_0$ : Maximum binding

5 The compound or a salt thereof obtained by the screening method or by the screening kit is a compound which changes the binding activity of a ligand polypeptide with a G protein-coupled receptor protein, wherein the compound inhibits or promotes the bonding, and, more particularly, it is a compound having a cell  
10 stimulating activity mediated via a G protein-coupled receptor or a salt thereof, so-called "G protein-coupled receptor agonist" or a compound having no said stimulating activity, so-called "G protein-coupled receptor antagonist". Examples of said compound are  
15 peptides, proteins, non-peptidic compounds, synthesized compounds, fermented products, etc. and the compound may be novel or known.

Said G protein coupled eceptor agonist has the same physiological action as the ligand to the G  
20 protein-coupled receptor protein has and, therefore, it is useful as a safe and less toxic pharmaceutical composition depending upon said ligand activity.

On the other hand, said G protein-coupled receptor antagonist is capable of inhibiting the physiological  
25 activity of the ligand to the G protein-coupled receptor protein and, therefore, it is useful as a safe and less toxic pharmaceutical composition for inhibiting said ligand activity.

The ligand polypeptide of the present invention  
30 relates to the pituitary function modulating action, central nervous system function modulating action or pancreatic function modulating action. Therefore, the above-mentioned agonist or antagonist can be used as a therapeutic and/or prophylactic agent for dementia such  
35 as senile dementia, cerebrovascular dementia (dementia due to cerebrovascular disorder), dementia associated



with phylodegenerative retroplastic diseases (e.g. Alzheimer's disease, Parkinson's disease, Pick's disease, Huntington's disease, etc.), dementia due to infectious diseases (e.g. delayed viral infections such as Creutzfeldt-Jakob disease), dementia associated with endocrine, metabolic, and toxic diseases (e.g. hypothyroidism, vitamin B12 deficiency, alcoholism, and poisoning due to various drugs, metals, or organic compounds), dementia associated with oncogenous diseases (e.g. brain tumor), dementia due to traumatic diseases (e.g. chronic subdural hematoma):, depression (melancholia), hyperkinetic (microencephalo-pathy) syndrome, disturbance of consciousness, anxiety syndrome, schizophrenia, horror, growth hormone secretory disease (e.g. gigantism, acromegalic gigantism etc.), hyperphagia, polyphagia, hypercholesterolemia, hyperglyceridemia, hyperlipemia, hyperprolactinemia, hypoglycemia, pituitarism, pituitary drawfism, diabetes (e.g. diabetic complications, diabetic nephropathy, diabetic neuropathy, diabetic retinopathy etc.), cancer (e.g. mammary cancer, lymphatic leukemia, cystic cancer, ovary cancer, prostatic cancer etc.), pancreatitis, renal disease (e.g. chronic renal failure, nephritis etc.), Turner's syndrome, neurosis, rheumatoid arthritis, spinal injury, transient brain ischemia, amyotrophic lateral sclerosis, acute myocardial infarction, spinocerebellar degeneration, bone fracture, trauma, atopic dermatitis, osteoporosis, asthma, epilepsy, infertility or oligogalactia.

Furthermore, the agonist or antagonist can be also used as hypnotic-sedative, agent for improvement in postoperative nutritional status, vasopressor or depressor.

When the compound or the salt thereof obtained by the screening method or by the screening kit is used as

the pharmaceutical composition, a conventional means which is the same means as using above-mentioned ligand polypeptide as the pharmaceutical composition may be applied therefor.

- 5 (5) Manufacture of Antibody or Antiserum against the Ligand Polypeptide or the G protein-coupled receptor Protein.

Antibodies, e.g. polyclonal antibody, monoclonal antibody, and antisera against the ligand polypeptide or the G protein-coupled receptor protein may be  
10 manufactured by antibody- or antiserum-manufacturing methods per se known to those of skill in the art or methods similar thereto, using the ligand polypeptide or the G protein-coupled receptor protein as antigen.  
15 For example, polyclonal antibodies can be manufactured by the method as given below.

[Preparation of a polyclonal antibody]

The above-mentioned polypeptide or protein as the  
20 antigen is coupled to a carrier protein. The carrier protein may for example be bovine thyroglobulin, bovine serum albumin, bovine gamma-globulin, hemocyanine, or Freund's complete adjuvant (Difco).

The coupling reaction between the antigen protein  
25 and the carrier protein can be carried out by the known procedure. The reagent for use in the coupling reaction includes but is not limited to glutaraldehyde and water-soluble carbodiimide. The suitable ratio of the antigen protein to the carrier protein is about 1:1  
30 through about 1:10 and as to the reaction pH, satisfactory results are obtained in many cases when the reaction is carried out around neutral, particularly in the range of pH about 6-8. The reaction time is preferably about 1 to 12 hours in many  
35 cases and more preferably about 2 to 6 hours. The conjugate thus obtained is dialyzed against water at

about 0 to 18°C in the routine manner and stored frozen or optionally lyophilized and stored.

For the production of a polyclonal antibody, a warm-blooded animal is inoculated with the immunogen produced in the manner described hereinbefore. The warm-blooded animal that can be used for this purpose includes mammalian warm-blooded animals, e.g. rabbit, sheep, goat, rat, mouse, guinea pig, bovine, equine, swine, etc.; and avian species, e.g. chicken, dove, duck, goose, quail, etc. Regarding the methodology for inoculating a warm-blooded animal with the immunogen, the inoculum size of the immunogen may be just sufficient for antibody production. For example, the desired antibody can be produced in many instances by emulsifying 1 mg of the immunogen in 1 ml of saline with Freund's complete adjuvant and injecting the emulsion subcutaneously at the back and hind-limb footpad of rabbits 5 times at 4-week intervals. For harvesting the antibody produced in the warm-blooded animal, for example a rabbit, the blood is withdrawn from the auricular vein usually during day 7 through day 12 after the last inoculation dose and centrifuged to recover an antiserum. For purification, the antiserum is generally subjected to affinity chromatography using a carrier to which each antigen peptide has been conjugated and the adsorbed fraction is recovered to provide a polyclonal antibody.

The monoclonal antibody can be produced by the following method.

#### [Preparation of Monoclonal Antibody]

##### (a) Preparation of Monoclonal Antibody-Producing Cells.

The ligand polypeptide or G protein-coupled receptor protein is administered to warm-blooded animals either solely or together with carriers or diluents to

the site where the production of antibody is possible by the administration. In order to potentiate the antibody productivity upon the administration, complete Freund's adjuvants or incomplete Freund's adjuvants may be administered. The administration is usually carried out once every two to six weeks and two to ten times in total. Examples of the applicable warm-blooded animals are monkeys, rabbits, dogs, guinea pigs, mice, rats, sheep, goats and chickens and the use of mice and rats is preferred.

In the preparation of the cells which produce monoclonal antibodies, an animal wherein the antibody titer is noted is selected from warm-blooded animals (e.g. mice) immunized with antigens, then spleen or lymph node is collected after two to five days from the final immunization and antibody-producing cells contained therein are fused with myeloma cells to give monoclonal antibody-producing hybridomas. Measurement of the antibody titer in antisera may, for example, be carried out by reacting a labeled ligand polypeptide or a labeled G protein-coupled receptor protein (which will be mentioned later) with the antiserum followed by measuring the binding activity of the labeling agent with the antibody. The operation for fusing may be carried out, for example, by a method of Koehler and Milstein (Nature, 256, 495, 1975), Examples of the fusion accelerator are polyethylene glycol (PEG), Sendai virus, etc. and the use of PEG is preferred.

Examples of the myeloma cells are NS-1, P3U1, SP2/0, AP-1, etc. and the use of P3U1 is preferred. The preferred fusion ratio of the numbers of antibody-producing cells used (spleen cells) to the numbers of myeloma cells is within a range of about 1:1 to 20:1. When PEG (preferably, PEG 1000 to PEG 6000) is added in a concentration of about 10-80% followed by incubating at 20-40°C (preferably, at 30-37°C) for one to ten

minutes, an efficient cell fusion can be carried out.

Various methods may be applied for screening a hybridoma which produces anti-ligand polypeptide antibody or anti-G protein-coupled receptor antibody.

- 5 For example, a supernatant liquid of hybridoma culture is added to a solid phase (e.g. microplate) to which the ligand polypeptide antigen or the G protein-coupled receptor protein antigen is adsorbed either directly or with a carrier, then anti-immunoglobulin antibody
- 10 (anti-mouse immunoglobulin antibody is used when the cells used for the cell fusion are those of mouse) which is labeled with a radioactive substance, an enzyme or the like, or protein A is added thereto and then anti-ligand polypeptide monoclonal antibodies or
- 15 anti-G protein-coupled receptor monoclonal antibodies bound on the solid phase are detected; or a supernatant liquid of the hybridoma culture is added to the solid phase to which anti-immunoglobulin or protein A is adsorbed, then the ligand polypeptide or the G protein-
- 20 coupled receptor labeled with a radioactive substance or an enzyme is added and anti-ligand polypeptide or anti-G protein-coupled receptor monoclonal antibodies bonded with the solid phase is detected.

- 25 Selection and cloning of the anti-ligand polypeptide monoclonal antibody- or the anti-G protein-coupled receptor monoclonal antibody-producing hybridoma may be carried out by methods per se known to those of skill in the art or methods similar thereto. Usually, it is carried out in a medium for animal
- 30 cells, containing HAT (hypoxanthine, aminopterin and thymidine). With respect to a medium for the selection, for the cloning and for the growth, any medium may be used so far as hybridoma is able to grow therein. Examples of the medium are an RPMI 1640
- 35 medium (Dainippon Pharmaceutical Co., Ltd., Japan) containing 1-20% (preferably 10-20%) of fetal calf

serum (FCS), a GIT medium (Wako Pure Chemical, Japan) containing 1-20% of fetal calf serum and a serum-free medium for hybridoma culturing (SFM-101; Nissui Seiyaku, Japan). The culturing temperature is usually 20-40°C and, preferably, about 37°C. The culturing time is usually from five days to three weeks and, preferably, one to two weeks. The culturing is usually carried out in 5% carbon dioxide gas. The antibody titer of the supernatant liquid of the hybridoma culture may be measured by the same manner as in the above-mentioned measurement of the antibody titer of the anti-ligand polypeptide or the anti-G protein-coupled receptor in the antiserum.

(b) Purification of the Monoclonal Antibody.

Like in the separation/purification of conventional polyclonal antibodies, the separation/purification of the anti-ligand polypeptide monoclonal antibody or the anti-G protein-coupled receptor monoclonal antibody may be carried out by methods for separating/purifying immunoglobulin such as salting-out, precipitation with an alcohol, isoelectric precipitation, electrophoresis, adsorption/deadsorption using ion exchangers such as DEAE, ultracentrifugation, gel filtration, specific purifying methods in which only an antibody is collected by treatment with an active adsorbent such as an antigen-binding solid phase, protein A or protein G and the bond is dissociated whereupon the antibody is obtained.

The ligand polypeptide antibody or the G protein-coupled receptor antibody which is manufactured by the aforementioned method (a) or (b) is capable of specifically recognizing ligand polypeptide or G protein-coupled receptors and, accordingly, it can be used for a quantitative determination of the ligand polypeptide or the G protein-coupled receptor in test liquid samples and particularly for a quantitative

determination by sandwich immunoassays.

Thus, the present invention provides, for example, the following methods:

- (i) a quantitative determination of a ligand polypeptide or a G protein-coupled receptor in a test liquid sample, which comprises
    - (a) competitively reacting the test liquid sample and a labeled ligand polypeptide or a labeled G protein-coupled receptor with an antibody which reacts with the ligand polypeptide or the G protein-coupled receptor, and
    - (b) measuring the ratio of the labeled ligand polypeptide or the labeled G protein-coupled receptor binding with said antibody; and
  - (ii) a quantitative determination of a ligand polypeptide or a G protein-coupled receptor in a test liquid sample, which comprises
    - (a) reacting the test liquid sample with an antibody immobilized on an insoluble carrier and a labeled antibody simultaneously or continuously, and
    - (b) measuring the activity of the labeling agent on the insoluble carrier
- wherein one antibody is capable of recognizing the N-terminal region of the ligand polypeptide or the G protein-coupled receptor while another antibody is capable of recognizing the C-terminal region of the ligand polypeptide or the G protein-coupled receptor.

When the monoclonal antibody of the present invention recognizing a ligand polypeptide or G protein-coupled receptor (hereinafter, may be referred to as "anti-ligand polypeptide or anti-G protein-coupled receptor antibody") is used, ligand polypeptide or G protein-coupled receptors can be measured and, moreover, can be detected by means of a tissue staining, etc. as well. For such an object, antibody molecules per se may be used or  $F(ab')_2$ , Fab' or Fab

fractions of the antibody molecule may be used too. There is no particular limitation for the measuring method using the antibody of the present invention and any measuring method may be used so far as it relates to a method in which the amount of antibody, antigen or antibody-antigen complex, depending on or corresponding to the amount of antigen, e.g. the amount of ligand polypeptide or G protein-coupled receptor, etc. in the liquid sample to be measured, is detected by a chemical or a physical means and then calculated using a standard curve prepared by a standard solution containing the known amount of antigen. For example, nephrometry, competitive method, immunometric method and sandwich method are suitably used and, in terms of sensitivity and specificity, the sandwich method which will be described herein later is particularly preferred.

Examples of the labeling agent used in the measuring method using the labeling substance are radioisotopes, enzymes, fluorescent substances, luminescent substances, colloids, magnetic substances, etc. Examples of the radioisotope are [ $^{125}\text{I}$ ], [ $^{131}\text{I}$ ], [ $^3\text{H}$ ] and [ $^{14}\text{C}$ ]; preferred examples of the enzyme are those which are stable and with big specific activity, such as  $\beta$ -galactosidase,  $\beta$ -glucosidase, alkali phosphatase, peroxidase and malate dehydrogenase; examples of the fluorescent substance are fluorescamine, fluorescein isothiocyanate, etc.; and examples of the luminescent substance are luminol, luminol derivatives, luciferin, lucigenin, etc. Further, a biotin-avidin system may also be used for binding an antibody or antigen with a labeling agent.

In an insolubilization (immobilization) of antigens or antibodies, a physical adsorption may be used or a chemical binding which is usually used for insolubilization or immobilization of proteins or



enzymes may be used as well. Examples of the carrier are insoluble polysaccharides such as agarose, dextran and cellulose; synthetic resins such as polystyrene, polyacrylamide and silicone; glass; etc.

5           In a sandwich (or two-site) method, the test liquid is made to react with an insolubilized anti-ligand polypeptide or anti-G protein-coupled receptor antibody (the first reaction), then it is made to react with a labeled anti-ligand polypeptide or a labeled  
10 anti-G protein-coupled receptor antibody (the second reaction) and the activity of the labeling agent on the insoluble carrier is measured whereupon the amount of the ligand polypeptide or the G protein-coupled receptor in the test liquid can be determined. The  
15 first reaction and the second reaction may be conducted reversely or simultaneously or they may be conducted with an interval. The type of the labeling agent and the method of insolubilization (immobilization) may be the same as those mentioned already herein. In the  
20 immunoassay by means of a sandwich method, it is not always necessary that the antibody used for the labeled antibody and the antibody for the solid phase is one type or one species but, with an object of improving the measuring sensitivity, etc., a mixture of two or  
25 more antibodies may be used too.

          In the method of measuring ligand polypeptide or G protein-coupled receptors by the sandwich method of the present invention, the preferred anti-ligand polypeptide antibodies or anti-G protein-coupled  
30 receptor antibodies used for the first and the second reactions are antibodies wherein their sites binding to the ligand polypeptide or the G protein-coupled receptors are different each other. Thus, the antibodies used in the first and the second reactions  
35 are those wherein, when the antibody used in the second reaction recognizes the C-terminal region of the ligand

polypeptide or the G protein-coupled receptor, then the antibody recognizing the site other than C-terminal regions, e.g. recognizing the N-terminal region, is preferably used in the first reaction.

5       The anti-ligand polypeptide antibody or the anti-G protein-coupled receptor antibody of the present invention may be used in a measuring system other than the sandwich method such as a competitive method, an immunometric method and a naphrometry. In a  
10 competitive method, an antigen in the test solution and a labeled antigen are made to react with an antibody in a competitive manner, then an unreacted labeled antigen (F) and a labeled antigen binding with an antibody (B) are separated (i.e. B/F separation) and the labeled  
15 amount of any of B and F is measured whereupon the amount of the antigen in the test solution is determined. With respect to a method for such a reaction, there are a liquid phase method in which a soluble antibody is used as the antibody and the B/F  
20 separation is conducted by polyethylene glycol, a second antibody to the above-mentioned antibody, etc.; and a solid phase method in which an immobilized antibody is used as the first antibody or a soluble antibody is used as the first antibody while an  
25 immobilized antibody is used as the second antibody.

In an immunometric method, an antigen in the test solution and an immobilized antigen are subjected to a competitive reaction with a certain amount of a labeled antibody followed by separating into solid and liquid  
30 phases; or the antigen in the test solution and an excess amount of labeled antibody are made to react, then a immobilized antigen is added to bind an unreacted labeled antibody with the solid phase and separated into solid and liquid phases. After that,  
35 the labeled amount of any of the phases is measured to determine the antigen amount in the test solution.

In a nephrometry, the amount of insoluble sediment which is produced as a result of the antigen-antibody reaction in a gel or in a solution is measured. Even when the antigen amount in the test solution is small and only a small amount of the sediment is obtained, a laser nephrometry wherein scattering of laser is utilized can be suitably used.

In applying each of those immunological measuring methods (immunoassays) to the measuring method of the present invention, it is not necessary to set up any special condition, operation, etc. therefor. A measuring system (assay system) for ligand polypeptide or G protein-coupled receptor may be constructed taking the technical consideration of the persons skilled in the art into consideration in the conventional conditions and operations for each of the methods. With details of those conventional technical means, a variety of reviews, reference books, etc. may be referred to. They are, for example, Hiroshi Irie (ed): "Radioimmunoassay" (Kodansha, Japan, 1974); Hiroshi Irie (ed): "Radioimmunoassay; Second Series" (Kodansha, Japan, 1979); Eiji Ishikawa et al. (ed): "Enzyme Immunoassay" (Igaku Shoin, Japan, 1978); Eiji Ishikawa et al. (ed): "Enzyme Immunoassay" (Second Edition) (Igaku Shoin, Japan, 1982); Eiji Ishikawa et al. (ed): "Enzyme Immunoassay" (Third Edition) (Igaku Shoin, Japan, 1987); "Methods in Enzymology" Vol. 70 (Immunochemical Techniques (Part A)); ibid. Vol. 73 (Immunochemical Techniques (Part B)); ibid. Vol. 74 (Immunochemical Techniques (Part C)); ibid. Vol. 84 (Immunochemical Techniques (Part D: Selected Immunoassays)); ibid. Vol. 92 (Immunochemical Techniques (Part E: Monoclonal Antibodies and General Immunoassay Methods)); ibid. Vol. 121 (Immunochemical Techniques (Part I: Hybridoma Technology and Monoclonal Antibodies)) (Academic Press); etc.

As such, the amount of ligand polypeptide or G protein-coupled receptor proteins can now be determined with a high precision using the anti-ligand polypeptide or the anti-G protein-coupled receptor antibody of the present invention.

In the specification and drawings of the present application, the abbreviations used for bases (nucleotides), amino acids and so forth are those recommended by the IUPAC-IUB Commission on Biochemical Nomenclature or those conventionally used in the art. Examples thereof are given below. Amino acids for which optical isomerism is possible are, unless otherwise specified, in the L form.

DNA : Deoxyribonucleic acid  
 15 cDNA : Complementary deoxyribonucleic acid  
 A : Adenine  
 T : Thymine  
 G : Guanine  
 C : Cytosine  
 20 RNA : Ribonucleic acid  
 mRNA : Messenger ribonucleic acid  
 dATP : Deoxyadenosine triphosphate  
 dTTP : Deoxythymidine triphosphate  
 dGTP : Deoxyguanosine triphosphate  
 25 dCTP : Deoxycytidine triphosphate  
 ATP : Adenosine triphosphate  
 EDTA : Ethylenediamine tetraacetic acid  
 SDS : Sodium dodecyl sulfate  
 EIA : Enzyme Immunoassay  
 30 G, Gly: Glycine (or Glycyl)  
 A, Ala: Alanine (or Alanyl)  
 V, Val: Valine (or Valyl)  
 L, Leu: Leucine (or Leucyl)  
 I, Ile: Isoleucine (or Isoleucyl)  
 35 S, Ser: Serine (or Seryl)  
 T, Thr: Threonine (or Threonyl)

- C, Cys: Cysteine (or Cysteinyl)  
 M, Met: Methionine (or Methionyl)  
 E, Glu: Glutamic acid (or Glutamyl)  
 D, Asp: Aspartic acid (or Aspartyl)  
 5 K, Lys: Lysine (or Lysyl)  
 R, Arg: Arginine (or Arginyl)  
 H, His: Histidine (or Histidyl)  
 F, Phe: Phenylalanine (or Phenylalanyl)  
 Y, Tyr: Tyrossine (or Tyrosyl)  
 10 W, Trp: Tryptophan (or Tryptophanyl)  
 P, Pro: Proline (or Prolyl)  
 N, Asn: Asparagine (or Asparaginyll)  
 Q, Gln: Glutamine (or Glutaminyl)  
 pGlu: Pyroglutamic acid (or Pyroglutamyl)  
 15 Me: Methyl  
 Et: Ethyl  
 Bu: Butyl  
 Ph: Phenyl  
 TC: Thiazolidinyl-4(R)-carboxamide  
 20 In this specification, substitutions, protective  
 groups and reagents commonly used are indicated by the  
 following abbreviations:  
 BHA : benzhydramine  
 pMBHA : p-methylbenzhydramine  
 25 Tos : p-toluenesulfonyl  
 CHO : formyl  
 HONB : N-hydroxy-5-norbornene-2,3-dicarboxyimide  
 OcHex : cyclohexyl ester  
 Bzl : benzyl  
 30 Bom : benzyloxymethyl  
 Br-Z : 2-bromobenzyloxycarbonyl  
 Boc : t-butoxycarbonyl  
 DCM : dichloromethane  
 HOBt : 1-hydroxybenztriazole  
 35 DCC : N,N'-dicyclohexylcarbodiimide  
 TFA : trifluoro acetate

DIEA : diisopropylethylamine  
 Fmoc : N-9-fluorenylmethoxycarbonyl  
 DNP : dinitrophenyl  
 Bum : t-butoxymethyl  
 5 Trt : trityl

Each SEQ ID NO set forth in the SEQUENCE LISTING  
 of the specification refers to the following sequence:  
 [SEQ ID NO:1] is an entire amino acid sequence of the  
 bovine pituitary-derived ligand polypeptide encoded by  
 10 the cDNA included in pBOV3.  
 [SEQ ID NO:2] is an entire nucleotide sequence of the  
 bovine pituitary-derived ligand polypeptide cDNA.  
 [SEQ ID NO:3] is an amino acid sequence of the bovine  
 pituitary-derived ligand polypeptide which was obtained  
 15 by purification and analysis of N-terminal sequence for  
 P-3 fraction. The amino acid sequence corresponds to  
 23rd to 51st positions of the amino acid sequence of  
 SEQ ID NO:1.  
 [SEQ ID NO:4] is an amino acid sequence of the bovine  
 pituitary-derived ligand polypeptide which was obtained  
 20 by purification and analysis of N-terminal sequence for  
 P-2 fraction. The amino acid sequence corresponds to  
 34th to 52nd positions of the amino acid sequence of  
 SEQ ID NO:1.  
 25 [SEQ ID NO:5] is an amino acid sequence of the bovine  
 pituitary-derived ligand polypeptide. The amino acid  
 sequence corresponds to 23rd to 53rd positions of the  
 amino acid sequence of SEQ ID NO:1.  
 [SEQ ID NO:6] is an amino acid sequence of the bovine  
 pituitary-derived ligand polypeptide. The amino acid  
 30 sequence corresponds to 23rd to 54th positions of the  
 amino acid sequence of SEQ ID NO:1.  
 [SEQ ID NO:7] is an amino acid sequence of the bovine  
 pituitary-derived ligand polypeptide. The amino acid  
 35 sequence corresponds to 23rd to 55th positions of the  
 amino acid sequence of SEQ ID NO:1.

5 [SEQ ID NO:9] is an amino acid sequence of the bovine  
pituitary-derived ligand polypeptide. The amino acid  
sequence corresponds to 34th to 54th positions of the  
amino acid sequence of SEQ ID NO:1.

10 [SEQ ID NO:10] is an amino acid sequence of the bovine  
pituitary-derived ligand polypeptide. The amino acid  
sequence corresponds to 34th to 55th positions of the  
amino acid sequence of SEQ ID NO:1.

15 [SEQ ID NO:11] is a nucleotide sequence of DNA coding  
for the bovine pituitary-derived ligand polypeptide  
(SEQ ID NO:3).

[SEQ ID NO:12] is a nucleotide sequence of DNA coding  
for the bovine pituitary-derived ligand polypeptide  
(SEQ ID NO:4).

20 [SEQ ID NO:13] is a nucleotide sequence of DNA coding  
for the bovine pituitary-derived ligand polypeptide  
(SEQ ID NO:5).

[SEQ ID NO:14] is a nucleotide sequence of DNA coding  
for the bovine pituitary-derived ligand polypeptide  
(SEQ ID NO:6).

25 [SEQ ID NO:15] is a nucleotide sequence of DNA coding  
for the bovine pituitary-derived ligand polypeptide  
(SEQ ID NO:7).

[SEQ ID NO:16] is a nucleotide sequence of DNA coding  
for the bovine pituitary-derived ligand polypeptide  
30 (SEQ ID NO:8).

[SEQ ID NO:17] is a nucleotide sequence of DNA coding  
for the bovine pituitary derived ligand polypeptide  
(SEQ ID NO:9).

[SEQ ID NO:18] is a nucleotide sequence of DNA coding  
35 for the bovine pituitary-derived ligand polypeptide  
(SEQ ID NO:10).

[SEQ ID NO:19] is a partial amino acid sequence of the human pituitary-derived G protein-coupled receptor protein encoded by the human pituitary-derived G protein-coupled receptor protein cDNA fragment included in p19P2.

[SEQ ID NO:20] is a partial amino acid sequence of the human pituitary-derived G protein-coupled receptor protein encoded by the human pituitary-derived G protein-coupled receptor protein cDNA fragment include in p19P2.

[SEQ ID NO:21] is an entire amino acid sequence of the human pituitary-derived G protein-coupled receptor protein encoded by the human pituitary-derived G protein-coupled receptor protein cDNA include in phGR3.

[SEQ ID NO:22] is a partial amino acid sequence of the mouse pancreatic  $\beta$ -cell line, MIN6-derived G protein-coupled receptor protein encoded by the mouse pancreatic  $\beta$ -cell line, MIN6-derived G protein-coupled receptor protein cDNA fragment having a nucleotide sequence (SEQ ID NO:27), derived based upon the nucleotide sequences of the mouse pancreatic  $\beta$ -cell line, MIN6-derived G protein-coupled receptor protein cDNA fragments each included in pG3-2 and pG1-10.

[SEQ ID NO:23] is a partial amino acid sequence of the mouse pancreatic  $\beta$ -cell line, MIN6-derived G protein-coupled receptor protein encoded by p5S38.

[SEQ ID NO:24] is a nucleotide sequence of the human pituitary-derived G protein-coupled receptor protein cDNA fragment include in p19P2.

[SEQ ID NO:25] is a nucleotide sequence of the human pituitary-derived G protein-coupled receptor protein cDNA fragment include in p19P2.

[SEQ ID NO:26] is an entire nucleotide sequence of the human pituitary-derived G protein-coupled receptor protein cDNA include in phGR3.

[SEQ ID NO:27] is a nucleotide sequence of the mouse



pancreatic  $\beta$ -cell line, MIN6-derived G protein-coupled receptor protein cDNA, derived based upon the nucleotide sequences of the mouse pancreatic  $\beta$ -cell line, MIN6-derived G protein-coupled receptor protein cDNA fragments each included in pG3-2 and pG1-10.

5 [SEQ ID NO: 28] is a nucleotide sequence of the mouse pancreatic  $\beta$ -cell line, MIN6-derived G protein-coupled receptor protein cDNA include in p5S38.

[SEQ ID NO:29] is a synthetic DNA primer for screening of cDNA coding for the G protein-coupled receptor protein.

10 [SEQ ID NO:30] is a synthetic DNA primer for screening of cDNA coding for the G protein-coupled receptor protein.

15 [SEQ ID NO:31] is a synthetic DNA primer for screening of cDNA coding for the G protein-coupled receptor protein.

[SEQ ID NO:32] is a synthetic DNA primer for screening of cDNA coding for the G protein-coupled receptor protein.

20 [SEQ ID NO:33] is a synthetic DNA primer for screening of cDNA coding for the G protein-coupled receptor protein.

[SEQ ID NO:34] is a synthetic DNA primer for screening of cDNA coding for the G protein-coupled receptor protein.

25 [SEQ ID NO:35] is a synthetic DNA primer for screening of cDNA coding for the bovine pituitary-derived ligand polypeptide, wherein the primer is represented by P5-1.

30 [SEQ ID NO:36] is a synthetic DNA primer for screening of cDNA coding for the bovine pituitary-derived ligand polypeptide, wherein the primer is represented by P3-1.

[SEQ ID NO:37] is a synthetic DNA primer for screening of cDNA coding for the bovine pituitary-derived ligand polypeptide, wherein the primer is represented by P3-2.

35 [SEQ ID NO:38] is a synthetic DNA primer for screening

of cDNA coding for the bovine pituitary-derived ligand polypeptide, wherein the primer is represented by PE. [SEQ ID NO:39] is a synthetic DNA primer for screening of cDNA coding for the bovine pituitary-derived ligand polypeptide, wherein the primer is represented by PDN. [SEQ ID NO:40] is a synthetic DNA primer for screening of cDNA coding for the bovine pituitary-derived ligand polypeptide, wherein the primer is represented by FB. [SEQ ID NO:41] is a synthetic DNA primer for screening of cDNA coding for the bovine pituitary-derived ligand polypeptide, wherein the primer is represented by FC. [SEQ ID NO:42] is a synthetic DNA primer for screening of cDNA coding for the bovine pituitary-derived ligand polypeptide, wherein the primer is represented by BOVF. [SEQ ID NO:43] is a synthetic DNA primer for screening of cDNA coding for the bovine pituitary-derived ligand polypeptide, wherein the primer is represented by BOVR. [SEQ ID NO:44] is an entire amino acid sequence of the bovine genome-derived ligand polypeptide. [SEQ ID NO: 45] is an entire amino acid sequence of the rat type ligand polypeptide encoded by the cDNA included in pRAV3. [SEQ ID NO:46] is an entire nucleotide sequence of the rat type ligand polypeptide cDNA. [SEQ ID NO:47] is an amino acid sequence of the rat type ligand polypeptide. The amino acid sequence corresponds to 22nd to 52nd positions of the amino acid sequence of SEQ ID NO:45. [SEQ ID NO:48] is an amino acid sequence of the rat type ligand polypeptide. The amino acid sequence corresponds to 22nd to 53rd positions of the amino acid sequence of SEQ ID NO:45. [SEQ ID NO:49] is an amino acid sequence of the rat type ligand polypeptide. The amino acid sequence corresponds to 22nd to 54th positions of the amino acid sequence of SEQ ID NO:45.

5 [SEQ ID NO:51] is an amino acid sequence of the rat type ligand polypeptide. The amino acid sequence corresponds to 33rd to 53rd positions of the amino acid sequence of SEQ ID NO:45.

[SEQ ID NO:53] is a nucleotide sequence encoding for the rat type ligand polypeptide of SEQ ID NO:47.

[SEQ ID NO:55] is a nucleotide sequence encoding for the rat type ligand polypeptide of SEQ ID NO:49.

[SEQ ID NO:57] is a nucleotide sequence encoding for the rat type ligand polypeptide of SEQ ID NO:51.

25 [SEQ ID NO:59] is an entire amino acid sequence of the  
human type ligand polypeptide encoded by the cDNA  
included in pHOB7.

30 [SEQ ID NO:61] is an amino acid sequence of the human  
type ligand polypeptide. The amino acid sequence  
corresponds to 23rd to 53rd positions of the amino acid  
sequence of SEQ ID NO.59.

35 [SEQ ID NO:62] is an amino acid sequence of the human  
type ligand polypeptide. The amino acid sequence  
corresponds to 23rd to 54th positions of the amino acid

sequence of SEQ ID NO.59.

[SEQ ID NO:63] is an amino acid sequence of the human type ligand polypeptide. The amino acid sequence corresponds to 23rd to 55th positions of the amino acid sequence of SEQ ID NO.59.

[SEQ ID NO:64] is an amino acid sequence of the human type ligand polypeptide. The amino acid sequence corresponds to 34th to 53rd positions of the amino acid sequence of SEQ ID NO.59.

[SEQ ID NO:65] is an amino acid sequence of the human type ligand polypeptide. The amino acid sequence corresponds to 34th to 54th positions of the amino acid sequence of SEQ ID NO.59.

[SEQ ID NO:66] is an amino acid sequence of the human type ligand polypeptide. The amino acid sequence corresponds to 34th to 55th positions of the amino acid sequence of SEQ ID NO.59.

[SEQ ID NO:67] is a nucleotide sequence encoding for the human type ligand polypeptide of SEQ ID NO:61.

[SEQ ID NO:68] is a nucleotide sequence encoding for the human type ligand polypeptide of SEQ ID NO:62.

[SEQ ID NO:69] is a nucleotide sequence encoding for the human type ligand polypeptide of SEQ ID NO:63.

[SEQ ID NO:70] is a nucleotide sequence encoding for the human type ligand polypeptide of SEQ ID NO:64.

[SEQ ID NO:71] is a nucleotide sequence encoding for the human type ligand polypeptide of SEQ ID NO:65.

[SEQ ID NO:72] is a nucleotide sequence encoding for the human type ligand polypeptide of SEQ ID NO:66.

[SEQ ID NO:73] is a partial amino acid sequence of the ligand polypeptide, wherein Xaa of the 10th position is Ala or Thr, Xaa of the 11th position is Gly or Ser and Xaa of the 21st position is H, Gly or GlyArg.

[SEQ ID NO:74] is a partial amino acid sequence of the ligand polypeptide, wherein Xaa of the 3rd position is Ala or Thr, Xaa of the 5th position is Gln or Arg and

Xaa of the 10th position is Ile or Thr.

[SEQ ID NO:75] is a synthetic DNA primer for screening of cDNA coding for the rat type ligand polypeptide, wherein the primer is represented by RA.

5 [SEQ ID NO:76] is a synthetic DNA primer for screening of cDNA coding for the rat type ligand polypeptide, wherein the primer is represented by RC.

[SEQ ID NO:77] is a synthetic DNA primer for screening of cDNA coding for the rat type ligand polypeptide, 10 wherein the primer is represented by rF.

[SEQ ID NO:78] is a synthetic DNA primer for screening of cDNA coding for the rat type ligand polypeptide, wherein the primer is represented by rR.

[SEQ ID NO:79] is a synthetic DNA primer for screening of cDNA coding for the human type ligand polypeptide, 15 wherein the primer is represented by R1.

[SEQ ID NO:80] is a synthetic DNA primer for screening of cDNA coding for the human type ligand polypeptide, wherein the primer is represented by R3.

20 [SEQ ID NO:81] is a synthetic DNA primer for screening of cDNA coding for the human type ligand polypeptide, wherein the primer is represented by R4.

[SEQ ID NO:82] is a synthetic DNA primer for screening of cDNA coding for the human type ligand polypeptide, 25 wherein the primer is represented by HA.

[SEQ ID NO:83] is a synthetic DNA primer for screening of cDNA coding for the human type ligand polypeptide, wherein the primer is represented by HB.

[SEQ ID NO:84] is a synthetic DNA primer for screening of cDNA coding for the human type ligand polypeptide, 30 wherein the primer is represented by HE.

[SEQ ID NO:85] is a synthetic DNA primer for screening of cDNA coding for the human type ligand polypeptide, wherein the primer is represented by HF.

35 [SEQ ID NO:86] is a synthetic DNA primer for screening of cDNA coding for the human type ligand polypeptide,

wherein the primer is represented by 5H.

[SEQ ID NO:87] is a synthetic DNA primer for screening of cDNA coding for the human type ligand polypeptide, wherein the primer is represented by 3HN.

5 [SEQ ID NO:88] is a synthetic DNA primer for screening of cDNA coding for the rat type G protein-coupled receptor protein (UHR-1), wherein the primer is represented by rRECF.

10 [SEQ ID NO:89] is a synthetic DNA primer for screening of cDNA coding for the rat type G protein-coupled receptor protein (UHR-1), wherein the primer is represented by rRECR.

[SEQ ID NO:90] is a synthetic DNA which is used for amplification of G3PDH, UHR-1 and ligand, wherein the primer represented by r19F.

[SEQ ID NO:91] is a synthetic DNA which is used for amplification of G3PDH, UHR-1 and ligand, wherein the primer represented by r19R.

20 [SEQ ID NO:92] is a N-terminal peptide of the ligand polypeptide, which is used for antigen. (Peptide-I)

[SEQ ID NO:93] is a C-terminal peptide of the ligand polypeptide, which is used for antigen. (Peptide-II)

[SEQ ID NO:94] is a peptide of the central portion in ligand polypeptide, which is used for antigen.

25 (Peptide-III)

[SEQ ID NO:95] is a synthetic DNA primer for screening of cDNA coding for rat type G protein-coupled receptor protein (UHR-1).

30 [SEQ ID NO:96] is a synthetic DNA primer for screening of cDNA coding for rat type G protein-coupled receptor protein (UHR-1).

The transformant *Escherichia coli*, designated INV $\alpha$ F'/p19P2, which is obtained in the Example 2 mentioned herein below, is on deposit under the terms of the Budapest Treaty from August 9, 1994, with the  
35 National Institute of Bioscience and Human-Technology

(NIBH), Agency of Industrial Science and Technology, Ministry of International Trade and Industry, Japan and has been assigned the Accession Number FERM BP-4776.

5 It is also on deposit from August 22, 1994 with the Institute for Fermentation, Osaka, Japan (IFO) and has been assigned the Accession Number IFO 15739.

10 The transformant *Escherichia coli*, designated INV $\alpha$ F'/pG3-2, which is obtained in the Example 4 mentioned herein below, is on deposit under the terms of the Budapest Treaty from August 9, 1994, with NIBH and has been assigned the Accession Number FERM BP-4775. It is also on deposit from August 22, 1994 with IFO and has been assigned the Accession Number IFO 15740.

15 The transformant *Escherichia coli*, designated JM109/phGR3, which is obtained in the Example 5 mentioned herein below, is on deposit under the terms of the Budapest Treaty from September 27, 1994, with NIBH and has been assigned the Accession Number FERM BP-4807. It is also on deposit from September 22, 1994 with IFO and has been assigned the Accession Number IFO 15748.

20 The transformant *Escherichia coli*, designated JM109/p5S38, which is obtained in the Example 8 mentioned herein below, is on deposit under the terms of the Budapest Treaty from October 27, 1994, with NIBH and has been assigned the Accession Number FERM BP-4856. It is also on deposit from October 25, 1994 with IFO and has been assigned the Accession Number IFO 15754.

30 The transformant *Escherichia coli*, designated JM109/pBOV3, which is obtained in the Example 20 mentioned herein below, is on deposit under the terms of the Budapest Treaty from February 13, 1996, with NIBH and has been assigned the Accession Number FERM BP-5391. It is also on deposit from January 25, 1996

with IFO and has been assigned the Accession Number IFO 15910.

5 The transformant *Escherichia coli*, designated JM109/pRAV3, which is obtained in the Example 29 mentioned herein below, is on deposit under the terms of the Budapest Treaty from September 12, 1996, with NIBH and has been assigned the Accession Number FERM BP-5665. It is also on deposit from September 3, 1996 with IFO and has been assigned the Accession Number IFO 10 16012.

15 The transformant *Escherichia coli*, designated JM109/pHOV7, which is obtained in the Example 32 mentioned herein below, is on deposit under the terms of the Budapest Treaty from September 12, 1996, with NIBH and has been assigned the Accession Number FERM BP-5666. It is also on deposit from September 5, 1996 with IFO and has been assigned the Accession Number IFO 16013.

20 [Industrial Application]

The bioactive substance of the present invention, namely the ligand polypeptide or its amide or ester thereof, or a salt thereof, a partial peptide thereof, or the DNA coding for said ligand polypeptide, has 25 function modulating activity for various tissues or internal organs, e.g. heart, lung, liver, spleen, thymus, kidney, adrenal glands, skeletal muscle, testis etc., besides pituitary, central nervous system or pancreas, and are useful as medicines. Furthermore, 30 the substance is useful for the screening of agonists or antagonists of G protein-coupled receptor proteins. The compounds which can be obtained by such screening also have function modulating activity for above-described tissues or internal organs, and are useful as 35 medicines.



## [Examples]

Described below are working examples of the present invention which are provided only for illustrative purposes, and not to limit the scope of the present invention.

## [Reference Example 1]

Preparation of Synthetic DNA Primer for Amplifying DNA Coding for G protein-coupled receptor Protein

A compariton of deoxyribonucleotide sequences coding for the known amino acid sequences corresponding to or near the first membrane-spanning domain each of human-derived TRH receptor protein (HTRHR), human-derived RANTES receptor protein (L10918, HUMRANTES), human Burkitt's lymphoma-derived unknown ligand receptor protein (X68149, HSBLR1A), human-derived somatostatin receptor protein (L14856, HUMSOMAT), rat-derived  $\mu$ -opioid receptor protein (U02083, RNU02083), rat-derived  $\kappa$ -opioid receptor protein (U00442, U00442), human-derived neuromedin B receptor protein (M73482, HUMNMBR), human-derived muscarinic acetylcholine receptor protein (X15266, HSHM4), rat-derived adrenaline  $\alpha_1$ B receptor protein (L08609, RATAADRE01), human-derived somatostatin 3 receptor protein (M96738, HUMSSTR3X), human-derived  $C_5a$  receptor protein (HUMC5AAR), human-derived unknown ligand receptor protein (HUMRDC1A), human-derived unknown ligand receptor protein (M84605, HUMOPIODRE) and rat-derived adrenaline  $\alpha_2$ B receptor protein (M91466, RATA2BAR) was made. As a result, highly homologous regions or parts were found.

Further, a comparison of deoxynucleotide sequences coding for the known amino acid sequences corresponding to or near the sixth membrane-spanning domain each of mouse-derived unknown ligand receptor protein (M80481, MUSGIR), human-derived bombesin receptor protein (L08893, HUMBOMB3S), human-derived adenosine A2

receptor protein (S46950, S46950), mouse-derived  
 unknown ligand receptor protein (D21061, MUSGPCR),  
 mouse-derived TRH receptor protein (S43387, S43387),  
 rat-derived neuromedin K receptor protein (J05189,  
 5 RATNEURA), rat-derived adenosine A1 receptor protein  
 (M69045, RATA1ARA), human-derived neurokinin A receptor  
 protein (M57414, HUMNEKAR), rat-derived adenosine A3  
 receptor protein (M94152, DATADENREC), human-derived  
 somatostatin 1 receptor protein (M81829, HUMSRI1A),  
 10 human-derived neurokinin 3 receptor protein (S86390,  
 S86371S4), rat-derived unknown ligand receptor protein  
 (X61496, RNCGPCR), human-derived somatostatin 4  
 receptor protein (L07061, HUMSSTR4Z) and rat-derived  
 GnRH receptor protein (M31670, RATGNRHA) was made. As  
 15 a result, highly homologous regions or parts were  
 found.

The aforementioned abbreviations in the  
 parentheses are identifiers (reference numbers) which  
 are indicated when GenBank/EMBL Data Bank is retrieved  
 20 by using DNASIS Gene/Protein Sequencing Data Base  
 (CD019, Hitachi Software Engineering, Japan) and are  
 usually called "Accession Numbers" or "Entry Names".  
 HTRHR is, however, the sequence as disclosed in  
 Japanese Patent Publication No. 304797/1993 (EPA  
 25 638645).

Specifically, it was planned to incorporate mixed  
 bases relying upon the base regions that were in  
 agreement with cDNAs coding for a large number of  
 receptor proteins in order to enhance base agreement of  
 30 sequences with as many receptor cDNAs as possible even  
 in other regions. Based upon these sequences, the  
 degenerate synthetic DNA having a nucleotide sequence  
 represented by SEQ ID NO:29 or SEQ ID NO:30 which is  
 complementary to the homologous nucleotide sequence  
 35 were produced.  
 [Synthetic DNAs]

5'-CGTGG (G or C) C (A or C) T (G or C) (G or C)  
TGGGCAAC (A, G, C or T) (C or T) CCTG-3'

(SEQ ID NO:29)

5'-GT (A, G, C or T) G (A or T) (A or G) (A or G) GGCA  
5 (A, G, C or T) CCAGCAGA (G or T) GGCAAA-3'

(SEQ ID NO:30)

The parentheses indicate the incorporation of a plurality of bases, leading to multiple oligonucleotides in the primer preparation. In other words, nucleotide residues in parentheses of the aforementioned DNAs were incorporated in the presence of a mixture of plural bases at the time of synthesis. [Example 1]

Amplification of Receptor cDNA by PCR Using Human Pituitary Gland-Derived cDNA

By using human pituitary gland-derived cDNA (QuickClone, CLONTECH Laboratories, Inc.) as a template, PCR amplification using the DNA primers synthesized in Reference Example 1 was carried out. The composition of the reaction solution consisted of the synthetic DNA primers (SEQ: 5' primer sequence and 3' primer sequence) each in an amount of 1  $\mu$ M, 1 ng of the template cDNA, 0.25 mM dNTPs, 1  $\mu$ l of Taq DNA polymerase and a buffer attached to the enzyme kit, and the total amount of the reaction solution was made to be 100  $\mu$ l. The cycle for amplification including 95°C for 1 min., 55°C for 1 min. and 72°C for 1 min. was repeated 30 times by using a Thermal Cycler (Perkin-Elmer Co.). Prior to adding Taq DNA polymerase, the remaining reaction solution was mixed and was heated at 95°C for 5 minutes and at 65°C for 5 minutes. The amplified products were confirmed relying upon 1.2% agarose gel electrophoresis and ethidium bromide staining.

[Example 2]

Subcloning of PCR Product into Plasmid Vector and

# Selection of Novel Receptor Candidate Clone via Decoding Nucleotide Sequence of Inserted cDNA Region

The PCR products were separated by using a 0.8%  
5 low-melting temperature agarose gel, the band parts  
were excised from the gel with a razor blade, and were  
heat-melted, extracted with phenol and precipitated in  
ethanol to recover DNAs. According to the protocol  
attached to a TA Cloning Kit (Invitrogen Co.), the  
10 recovered DNAs were subcloned into the plasmid vector,  
pCR<sup>TM</sup> II (TM represents registered trademark). The  
recombinant vectors were introduced into E. coli INVαF'  
competent cells (Invitrogen Co.) to produce  
transformants. Then, transformant clones having a  
15 cDNA-inserted fragment were selected in an LB agar  
culture medium containing ampicillin and X-gal. Only  
transformant clones exhibiting white color were picked  
with a sterilized toothstick to obtain transformant  
Escherichia coli INVαF'/p19P2.

20 The individual clones were cultured overnight in  
an LB culture medium containing ampicillin and treated  
with an automatic plasmid extracting machine (Kurabo  
Co., Japan) to prepare plasmid DNAs. An aliquot of the  
DNA thus prepared was cut by EcoRI to confirm the size  
25 of the cDNA fragment that was inserted. An aliquot of  
the remaining DNA was further processed with RNase,  
extracted with phenol/chloroform, and precipitated in  
ethanol so as to be condensed. Sequencing was carried  
out by using a DyeDeoxy terminator cycle sequencing kit  
30 (ABI Co.), the DNAs were decoded by using a fluorescent  
automatic sequencer, and the data of the nucleotide  
sequences obtained were read by using DNASIS (Hitachi  
System Engineering Co., Japan). The underlined  
portions represent regions corresponding to the  
35 synthetic primers.

Homology retrieval was carried out based upon the

determined nucleotide sequences [SEQ ID NO:24 and 25  
(Here, the determined nucleotide sequence is the  
nucleotide sequence which the underlined portion is  
deleted from the sequence of Figure 1 or Figure 2  
5 respectively)].

As a result, it was learned that a novel G  
protein-coupled receptor protein was encoded by the  
cDNA fragment insert in the plasmid, p19P2, possessed  
by the transformant Escherichia coli INV $\alpha$ F'/p19P2. To  
10 further confirm this fact, by using DNASIS (Hitachi  
System Engineering Co., Japan) the nucleotide sequences  
were converted into amino acid sequences [SEQ ID NO:19  
and 20], and homology retrieval was carried out in view  
of hydrophobicity plotting [Figures 3 and 4] and at the  
15 amino acid sequence level to find homology relative to  
neuropeptide Y receptor proteins [Figure 5].  
[Example 3]

Preparation of Poly(A)<sup>+</sup>RNA Fraction from Mouse  
Pancreatic  $\beta$ -Cell Strain, MIN6 and Synthesis of  
20 cDNA

A total RNA was prepared from the mouse pancreatic  
 $\beta$ -cell strain, MIN6 (Jun-ichi Miyazaki et al.,  
Endocrinology, Vol. 127, No. 1, p.126-132) according to  
the guanidine thiocyanate method (Kaplan B.B. et al.,  
25 Biochem. J., 183, 181-184 (1979) and, then, poly(A)<sup>+</sup>RNA  
fractions were prepared with a mRNA purifying kit  
(Pharmacia Co.). Next, to 5  $\mu$ g of the poly(A)<sup>+</sup>RNA  
fraction was added a random DNA hexamer (BRL Co.) as a  
primer, and the resulting mixture was subjected to  
30 reaction with mouse Moloney Leukemia virus (MMLV)  
reverse transcriptase (BRL Co.) in the buffer attached  
to the MMLV reverse transcriptase kit to synthesize  
complementary DNAs. The reaction product was extracted  
with phenol/chloroform (1:1), precipitated in ethanol,  
35 and was then dissolved in 30  $\mu$ l of TE buffer (10 mM  
Tris-HCL at pH8.0, 1 mM EDTA at pH8.0).

## [Example 4]

## Amplification of Receptor cDNA by PCR Using MIN6-Derived cDNA and Sequencing

By using, as a template, 5  $\mu$ l of cDNA prepared from the mouse pancreatic  $\beta$ -cell strain, MIN6 in the above Example 3, PCR amplification using the DNA primers synthesized in Reference Example 1 was carried out under the same condition as in Example 1. The resulting PCR product was subcloned into the plasmid vector, pCR<sup>TM</sup>II, in the same manner as in Example 2 to obtain a plasmid, pG3-2. The plasmid pG3-2 was transfected into E. coli INV $\alpha$ F' to obtain transformed Escherichia coli INV $\alpha$ F'/pG3-2.

By using, as a template, 5  $\mu$ l of the cDNA prepared from the mouse pancreatic  $\beta$ -cell strain, MIN6, PCR amplification using DNA primers as disclosed in Libert F. et al., "Science, 244:569-572, 1989", i.e., a degenerate synthetic primer represented by the following sequence:

5'-CTGTG (C or T) G (C or T) (G or C) AT (C or T) GCIIT  
(G or T) GA (C or T) (A or C) G (G or C) TAC-3'

(SEQ ID NO:31)

wherein I is inosine; and  
a degenerate synthetic primer represented by the following sequence:

5'-A (G or T) G (A or T) AG (A or T) AGGGCAGCCAGCAGAI  
(G or C) (A or G) (C or T) GAA-3'

(SEQ ID NO:32)

wherein I is inosine,  
was carried out under the same conditions as in Working Example 1. The resulting PCR product was subcloned into the plasmid vector, pCR<sup>TM</sup>II, in the same manner as described in Example 2 to obtain a plasmid, pG1-10.

The reaction for determining the nucleotide sequence (sequencing) was carried out with a DyeDeoxy terminator cycle sequencing kit (ABI Co.), the DNA was

decoded with the fluorescent automatic sequencer (ABI Co.), and the data of the nucleotide sequence obtained were analyzed with DNASIS (Hitachi System Engineering Co., Japan).

5        Figure 6 shows a mouse pancreatic  $\beta$ -cell strain MIN6-derived G protein-coupled receptor protein-encoding DNA (SEQ ID NO:27) and an amino acid sequence (SEQ ID NO:22) encoded by the isolated DNA based upon the nucleotide sequences of plasmids pG3-2 and pG1-10  
10        which are held by the transformant Escherichia coli INV $\alpha$ F'/pG3-2. The underlined portions represent regions corresponding to the synthetic primers.

         Homology retrieval was carried out based upon the determined nucleotide sequence [Figure 6]. As a  
15        result, it was learned that a novel G protein-coupled receptor protein was encoded by the cDNA fragment obtained. To further confirm this fact, by using DNASIS (Hitachi System Engineering Co., Japan) the nucleotide sequence was converted into an amino acid  
20        sequence [Figure 6], hydrophobicity plotting was carried out to confirm the presence of six hydrophobic regions [Figure 8]. Upon comparing the amino acid sequence with that of p19P2 obtained in Example 2, furthermore, a high degree of homology was found as  
25        shown in [Figure 7]. As a result, it is strongly suggested that the G protein-coupled receptor proteins encoded by pG3-2 and pG1-10 recognize the same ligand as the G protein-coupled receptor protein encoded by p19P2 does while the animal species from which the  
30        receptor proteins encoded by pG3-2 and pG1-10 are derived is different from that from which the receptor protein encoded by p19P2 is.

[Example 5]

35        Cloning of cDNA Comprising Whole Coding Regions for Receptor Protein from Human Pituitary Gland-Derived cDNA Library

The DNA library constructed by Clontech Co. wherein  $\lambda$  gt11 phage vector is used (CLONTECH Laboratories, Inc.; CLH L1139b) was employed as a human pituitary gland-derived cDNA library. The human  
 5 pituitary gland cDNA library ( $2 \times 10^6$  pfu (plaque forming units)) was mixed with E. coli Y1090<sup>-</sup> treated with magnesium sulfate, and incubated at 37°C for 15 minutes followed by addition of 0.5% agarose (Pharmacia Co.) LB. The E. coli was plated onto a 1.5% agar  
 10 (Wako-Junyaku Co.) LB plate (containing 50  $\mu$ g/ml of ampicillin). A nitrocellulose filter was placed on the plate on which plaques were formed and the plaque was transferred onto the filter. The filter was denatured with an alkali and then heated at 80°C for 3 hours to  
 15 fix DNAs.

The filter was incubated overnight at 42°C together with the probe mentioned herein below in a buffer containing 50% formamide, 5 x SSPE (20 x SSPE (pH 7.4) is 3 M NaCl, 0.2 M  $\text{NaH}_2\text{PO}_4 \cdot \text{H}_2\text{O}$ , 25 mM EDTA), 5  
 20 X Denhardt's solution (Nippon Gene, Japan), 0.1% SDS and 100  $\mu$ g/ml of salmon sperm DNA for hybridization.

The probe used was obtained by cutting the DNA fragment inserted in the plasmid, p19P2, obtained in Working Example 2, with EcoRI, followed by recovery and  
 25 labelling by incorporation of [ $^{32}$ P]dCTP (Dupont Co.) with a random prime DNA labelling kit (Amasham Co.).

It was washed with 2 x SSC (20 x SSC is 3 M NaCl, 0.3 M sodium citrate), 0.1% SDS at 55°C for 1 hour and, then, subjected to an autoradiography at -80°C to  
 30 detect hybridized plaques.

In this screening, hybridization signals were recognized in three independent plaques. Each DNA was prepared from the three clones. The DNAs digested with EcoRI were subjected to an agarose electrophoresis and  
 35 were analyzed by the southern blotting using the same probe as the one used in the screening. Hybridizing



bands were identified at about 0.7kb, 0.8kb and 2.0kb, respectively. Among them, the DNA fragment corresponding to the band at about 2.0kb ( $\lambda$  hGR3) was selected. The  $\lambda$  hGR3-derived EcoRI fragment with a hybridizable size was subcloned to the EcoRI site of the plasmid, pUC18, and *E. coli* JM109 was transformed with the plasmid to obtain transformant *E. coli* JM109/phGR3. A restriction enzyme map of the plasmid, phGR3, was prepared relying upon a restriction enzyme map deduced from the nucleotide sequence as shown in Example 2. As a result, it was learned that it carried a full-length receptor protein-encoding DNA which was predicted from the receptor protein-encoding DNA as shown in Example 2.

[Example 6]

#### Sequencing of Human Pituitary Gland-Derived Receptor Protein cDNA

Among the EcoRI fragments inserted in the plasmid, phGR3, obtained in the above Example 5, the from EcoRI to NheI nucleotide sequence with about 1330bp that is considered to be a receptor protein-coding region was sequenced. Concretely speaking, by utilizing restriction enzyme sites that exist in the EcoRI fragments, unnecessary parts were removed or necessary fragments were subcloned in order to prepare template plasmids for analyzing the nucleotide sequence.

The reaction for determining the nucleotide sequence (sequencing) was carried out with a DyeDeoxy terminator cycle sequencing kit (ABI Co.), the DNA was decoded with the fluorescent automatic sequencer (ABI Co.), and the data of the nucleotide sequence obtained were analyzed with DNASIS (Hitachi System Engineering Co., Japan).

Figure 9 shows a nucleotide sequence of from immediate after the EcoRI site up to the NheI site encoded by phGR3. The nucleotide sequence of the human

pituitary gland-derived receptor protein-encoding DNA corresponds to the nucleotide sequence (SEQ ID NO:26) of from 118th to 1227th nucleotides [Figure 9]. An amino acid sequence of the receptor protein that is encoded by the nucleotide sequence is shown in SEQ ID NO:21.

[Example 7]

Northern Hybridization with Human Pituitary Gland-Derived Receptor Protein-Encoding phGR3

Northern blotting was carried out in order to detect the expression of phGR3-encoded human pituitary gland-derived receptor proteins obtained in Example 5 in the pituitary gland at a mRNA level. Human pituitary gland mRNA (2.5 µg, Clontech Co.) was used as a template mRNA and the same as the probe used in Working Example 5 was used as a probe. Nylon membrane (Pall Biodyne, U.S.A.) was used as a filter for northern blotting and migration of the mRNA and adsorption (sucking) thereof with the blotting filter was carried out according to the method as disclosed in Molecular Cloning, Cold Spring Harbor Laboratory Press, 1989.

The hybridization was effected by incubating the above-mentioned filter and probe in a buffer containing 50% formamide, 5 x SSPE, 5 X Denhardt's solution, 0.1% SDS and 100 µg/ml of salmon sperm DNA overnight at 42°C. The filter was washed with 0.1 x SSC, 0.1% SDS at 50°C and, after drying with an air, was exposed to an X-ray film (XAR5, Kodak) for three days at -80°C. The results were as shown in Figure 10 from which it is considered that the receptor gene encoded by phGR3 is expressed in the human pituitary gland.

[Example 8]

Amplification of Receptor cDNA by PCR Using MIN6-Derived cDNA and Sequencing

By using, as a template, 5 µl of cDNA prepared

from the mouse pancreatic  $\beta$ -cell strain, MIN6 in Working Example 3, PCR amplification using the DNA primers synthesized in Example 4 as disclosed in Libert F. et al., "Science, 244:569-572, 1989", i.e., a

5 synthetic primer represented by the following sequence:  
 5'-CTGTG (C or T) G (C or T) (G or C) AT (C or T) GCIIT  
 (G or T) GA (C or T) (A or C) G (G or C) TAC-3'  
 (SEQ ID NO:31)

wherein I is inosine; and

10 a synthetic primer represented by the following sequence:

5'-A (G or T) G (A or T) AG (A or T) AGGGCAGCCAGCAGAI  
 (G or C) (A or G) (C or T) GAA-3'  
 (SEQ ID NO:32)

15 wherein I is inosine, was carried out under the same conditions as in Example 1. The resulting PCR product was subcloned to the plasmid vector, pCR<sup>TM</sup>II, in the same manner as in Example 2 to obtain a plasmid, p5S38. The plasmid p5S38 was transfected into E. coli JM109 to  
 20 obtain transformant Escherichia coli JM109/p5S38.

The reaction for determining the nucleotide sequence (sequencing) was carried out with a DyeDeoxy terminator cycle sequencing kit (ABI Co.), the DNA was decoded with the fluorescent automatic sequencer (ABI  
 25 Co.), and the data of the nucleotide sequence obtained were read with DNASIS (Hitachi System Engineering Co., Japan).

Figure 12 shows a mouse pancreatic  $\beta$ -cell strain MIN6-derived G protein-coupled receptor protein-  
 30 encoding DNA (SEQ ID NO:28) and an amino acid sequence (SEW ID NO:23) encoded by the isolated DNA based upon the nucleotide sequence of plasmid, p5S38. The underlined portions represent regions corresponding to the synthetic primers.

35 Homology retrieval was carried out based upon the determined nucleotide sequence [Figure 12]. As a

result, it was learned that a novel G protein-coupled receptor protein was encoded by the cDNA fragment obtained. To further confirm this fact, by using DNASIS (Hitachi System Engineering Co., Japan), the nucleotide sequence was converted into an amino acid sequence [Figure 12], and hydrophobicity plotting was carried out to confirm the presence of four hydrophobic regions [Figure 14]. Upon comparing the amino acid sequence with those encoded by p19P2 obtained in Example 2 and encoded by pG3-2 obtained in Example 4, furthermore, a high degree of homology was found as shown in Figure 13. As a result, it is strongly suggested that the mouse pancreatic  $\beta$ -cell strain, MIN6-derived G protein-coupled receptor protein encoded by p5S38 recognizes the same ligand as the human pituitary gland-derived G protein-coupled receptor protein encoded by p19P2 does while the animal species from which the receptor protein encoded by p5S38 is derived is different from that from which the receptor protein encoded by p19P2 is. It is also strongly suggested that the mouse pancreatic  $\beta$ -cell strain, MIN6-derived G protein-coupled receptor protein encoded by p5S38 recognized the same ligand as the mouse pancreatic  $\beta$ -cell strain, MIN6-derived G protein-coupled receptor proteins encoded by pG3-2 and pG1-10 do and they are analogous receptor proteins one another (so-called "subtype").

[Example 9]

Preparation of CHO cells which express phGR3

The plasmid phGR3 (Example 5) containing a cDNA encoding the full-length amino acid sequence of human pituitary receptor protein was digested with the restriction enzyme Nco I and electrophoresed on agarose gel and a fragment of about 1kb was recovered. Both ends of the recovered fragment were blunted with a DNA blunting kit (Takara Shuzo Co., Japan) and, with the

SalI linker added, treated with SalI and inserted into the SalI site of pUC119 to provide plasmid S10. Then, S10 was treated with SalI and SacII to prepare a fragment of about 700 bp (containing the N-terminal coding region). Then, a fragment of about 700 bp (containing the C-terminal coding region including initiation and termination codons) was cut out from phGR3 with Sac II and Nhe I. These two fragments were added to the animal cell expression vector plasmid pAKKO-111H (the vector plasmid identical to the pAKKO1.11 H described in Biochim. Biophys. Acta, Hinuma, S., et al., 1219 251-259, 1994) and a ligation reaction was carried out to construct a full-length receptor protein expression plasmid pAKKO-19P2.

E. coli transfected with pAKKO-19P2 was cultured and the pAKKO-19P2 plasmid DNA was mass-produced using QUIAGEN Maxi. A 20 µg portion of the plasmid DNA was dissolved in 1 ml of sterile PBS, and in a gene transfer vial (Wako Pure Chemical Ind.), the solution was vortexed well for liposome formation. This liposome, 125 µl, was added to CHOdhfr<sup>-</sup> cells subcultured at  $1 \times 10^6$  per 10cm-dia. dish 24 hr before and placed in fresh medium immediately before addition and overnight culture was carried out. After a further one-day culture in fresh medium, the medium was changed to a screening medium and the incubation was further carried out for a day. For efficient screening of transformants, subculture was carried out at a low cell density and only the cells growing in the screening medium were selected to establish a full-length receptor protein expression CHO cell line CHO-19P2.

[Example 10]

Confirmation of the amount of expression of the full-length receptor protein in the CHO-19P2 cell line at the transcription level

Using FastTrack Kit (Invitrogen), CHO cells

transfected with pAKKO-19P2 according to the kit manual and mock CHO cells were used to prepare poly(A)<sup>+</sup>RNA. Using 0.02 µg of this poly(A)<sup>+</sup>RNA, a cDNA was synthesized by means of RNA PCR Kit (Takara Shuzo, Co., Japan). The kind of primer used was a random 9mer and the total volume of the reaction mixture was 40 µl. As a negative control of cDNA synthesis, a reverse transcriptase-free reaction mixture was also provided. First, the reaction mixture was incubated at 30°C for 10 minutes to conduct an amplification reaction to some extent. Then, it was incubated at 42°C for 30 minutes to let the reverse transcription reaction proceed. The enzyme was inactivated by heating at 99°C for 5 minutes and the reaction system was cooled at 5°C for 5 minutes.

After completion of the reverse transcription reaction, a portion of the reaction mixture was recovered and after dilution with distilled water, extraction was carried out with phenol/chloroform and further with diethyl ether. The extract was subjected to precipitation from ethanol and the precipitate was dissolved in a predetermined amount of distilled water for use as a cDNA sample. This cDNA solution and the plasmid DNA (pAKKO-19P2) were serially diluted and using primers specific to full-length receptor protein, PCR was carried out. The sequences of the primers prepared according to the base sequence of the coding region of the full-length receptor protein were CTGACTTATTTTCTGGGCTGCGC (SEQ ID NO:33) for 5' end and AACACCGACACATAGACGGTGACC (SEQ ID NO:34) for 3' end.

The PCR reaction was carried out in a total volume of 100 µl using 1 µM each of the primers, 0.5 µl of Taq DNA polymerase (Takara Shuzo Co., Japan), the reaction buffer and dNTPs accompanying the enzyme, and 10 µl of template DNA (cDNA or plasmid solution). First the reaction mixture was heat-treated at 94°C for 2 minutes

for sufficient denaturation of the template DNA and subjected to 25 cycles of 95°C x 30 seconds, 65°C x 30 seconds, and 72°C x 60 seconds. After completion of the reaction, 10 µl of the reaction mixture was subjected to agarose gel electrophoresis and the detection and quantitative comparison of amplification products were carried out. As a result, a PCR product of the size (400 bp) predictable from the sequence of the cDNA coding for the full-length receptor protein was detected [Fig. 15]. In the lane of the PCR reaction mixture using the product of the reverse transcriptase-free transcription system as the template, no specific band was detected, thus extruding the possibility of its being a PCR product derived from the genomic DNA of CHO cells. Moreover, no specific band appeared in the lane of mock cells, either. Therefore, it was clear that the product was not derived from the mRNA initially expressed in CHO cells [Fig. 15].

[Example 11]

20       Detection of the activity to specifically promote release of arachidonic acid metabolites from CHO-19P2 cells in a rat whole brain extract

A crude peptide fraction was prepared from rat whole brain by the following procedure. The rat whole brain enucleated immediately after sacrifice was frozen in liquefied nitrogen and stored at -80°C. The frozen rat whole brain, 20 g (the equivalent of 10 rats) was finely divided and boiled in 80 ml of distilled water for 10 minutes. After the boiled tissue was quenched on ice, 4.7 ml of acetic acid was added at a final concentration of 1.0 M and the mixture was homogenized using a Polytron (20,000 rpm, 6 min.). The homogenate was stirred overnight and then centrifuged (10,000 rpm, 20 min.) to separate the supernatant. The sediment was homogenized in 40 ml of 1.0 M acetic acid and centrifuged again to recover the supernatant. The

supernatants were pooled, diluted in 3 volumes of acetone, allowed to stand on ice for 30 minutes, and centrifuged (10,000 rpm, 20 min.) to recover the supernatant. The recovered supernatant was evaporated to remove acetone. To the resulting acetone-free concentrate was added 2 volumes of 0.05% trifluoroacetic acid(TFA)/H<sub>2</sub>O and the mixture was applied to a reversed-phase C18 column (Prep C18 125Å, Millipore). After application of the supernatant, the column was washed with 0.05% TFA/H<sub>2</sub>O, and gradient elution was carried out with 10%, 20%, 30%, 40%, 50%, and 60% CH<sub>3</sub>CN/0.05%TFA/H<sub>2</sub>O. The fractions were respectively divided into 10 equal parts and lyophilized. The dried sample derived from one animal equivalent of rat whole brain was dissolved in 20 µl of dimethyl sulfoxide (DMSO) and suspended in 1/ml of Hank's balanced saline solution (HBSS) supplemented with 0.05% bovine serum albumin (BSA) to provide a crude peptide fraction.

The full-length receptor protein-expressed CHO cells and mock CHO cells were seeded in a 24-well plate,  $0.5 \times 10^5$  cells/well, and cultured for 24 hours. Then, [<sup>3</sup>H] arachidonic acid was added at a final concentration of 0.25µCi/well. Sixteen (16) hours after addition of [<sup>3</sup>H] arachidonic acid, the cells were rinsed with 0.05% BSA-HBSS and the above-mentioned crude peptide fraction was added, 400 µl/well. The mixture was incubated at 37°C for 30 minutes and a 300 µl portion of the reaction mixture (400 µl) was added to 4 ml of a scintillator and the amount of [<sup>3</sup>H] arachidonic acid metabolite released into the reaction mixture was determined with a scintillation counter. As a result, an arachidonic acid metabolite-releasing activity specific to the full-length receptor protein expressed CHO cells (CHO-19P2) was detected in the 30%



CH<sub>3</sub>CN fraction of the eluate [Fig. 16].

[Example 12]

5           Detection of the activity to specifically promote  
          release of arachidonic acid metabolites from CHO-  
          19P2 cells in a bovine hypothalamus extract

          A crude peptide fraction was prepared from 360 g  
(the equivalent of 1 animals) of bovine brain tissue  
including hypothalamus in the same manner as in Example  
11. A dried peptide sample per 0.05 animal was  
10       dissolved in 40 µl of DMSO and suspended in 2 ml of  
          0.05% BSA-HBSS and the detection of arachidonic acid  
          metabolite-releasing activity was attempted in the same  
          manner as in Example 11. As a result, the activity to  
          specifically promote release of arachidonic acid  
15       metabolites from the CHO-19P2 cell line was detected in  
          the fraction eluted with 30% CH<sub>3</sub>CN from a C18 column to  
          which the crude bovine hypothalamus peptide fraction  
          had been applied [Fig. 17].

[Example 13]

20           Preparation of the activity (peptide) to  
          specifically promote release of arachidonic acid  
          metabolites from CHO-19P2 cells by purification  
          from bovine hypothalamus

          A typical process for harvesting the activity to  
25       specifically promote release of arachidonic acid  
          metabolites from the CHO-19P2 cell line by purification  
          from bovine hypothalamus is now described. A frozen  
          bovine brain tissue specimen including hypothalamus,  
          4.0 kg (the equivalent of 80 animals) was ground and  
30       boiled in 8.0 L of distilled water for 20 minutes.  
          After quenching on ice, 540 ml of acetic acid was added  
          at a final concentration of 1.0 M and the mixture was  
          homogenized using a Polytron (10,000 rpm, 12 min.).  
          The homogenate was stirred overnight and then  
35       centrifuged (9,500 rpm, 20 min) to recover a  
          supernatant. The sediment was suspended in 4.0 L of

1.0 M acetic acid and homogenized with the Polytron and centrifuged again to recover a further supernatant. The supernatants were pooled and TFA was added at a final concentration of 0.05%. The mixture was applied to reversed-phase C18 (Prep C18 125Å, 160 ml; Millipore) packed in a glass column. After addition, the column was washed with 320 ml of 0.05% TFA/H<sub>2</sub>O and 3-gradient elution was carried out with 10%, 30%, and 50% CH<sub>3</sub>CN/0.05% TFA/H<sub>2</sub>O. To the 30% CH<sub>3</sub>CN/0.05% TFA/H<sub>2</sub>O fraction was added 2 volumes of 20 mM CH<sub>3</sub>COONH<sub>4</sub>/H<sub>2</sub>O and the mixture was applied to the cation exchange column HiPrep CM-Sepharose FF (Pharmacia). After the column was washed with 20 mM CH<sub>3</sub>COONH<sub>4</sub>/10% CH<sub>3</sub>CN/H<sub>2</sub>O, 4-gradient elution was carried out with 100 mM, 200 mM, 500 mM, and 1000 mM CH<sub>3</sub>COONH<sub>4</sub>/10% CH<sub>3</sub>CN/H<sub>2</sub>O. In the 200 mM CH<sub>3</sub>COONH<sub>4</sub> fraction, activity to specifically promote release of arachidonic acid metabolites from CHO-19P2 was detected. Therefore, this fraction was diluted with 3 volumes of acetone, centrifuged for deproteination, and concentrated in an evaporator. To the concentrated fraction was added TFA (final concentration 0.1%) and the mixture was adjusted to pH 4 with acetic acid and applied to 3 ml of the reversed-phase column RESOURCE RPC (Pharmacia). Elution was carried out on a concentration gradient of 15%-30% CH<sub>3</sub>CN. As a result, activity to specifically promote the release of arachidonic acid metabolites from the CHO-19P2 cell line was detected in the 19%-21% CH<sub>3</sub>CN fraction. The active fraction eluted from RESOURCE RPC was lyophilized, dissolved with DMSO, suspended in 50 mM MES pH 5.0/10% CH<sub>3</sub>CN, and added to 1 ml of the cation exchange column RESOURCE S. Elution was carried out on a concentration gradient of 0 M-0.7 M NaCl. As a result, the activity to specifically promote release of arachidonic acid metabolites from CHO-19P2 cells was

detected in the 0.32 M-0.46 M NaCl fraction. The active eluate from RESOURCE S was lyophilized, dissolved with DMSO, suspended in 0.1% TFA/H<sub>2</sub>O, and added to reversed-phase column C18 218TP5415 (Vydac), and elution was carried out on a concentration gradient of 20%-30% CH<sub>3</sub>CN. As a result, the activity to specifically promote release of arachidonic acid metabolites from CHO-19P2 cells was detected in the three fractions 22.5%, 23%, and 23.5% CH<sub>3</sub>CN (these active fractions are designated as P-1, P-2, and P-3) [Fig. 18]. Of the three active fractions, the 23.5% CH<sub>3</sub>CN fraction (P-3) was lyophilized, dissolved with DMSO, suspended in 0.1% TFA/H<sub>2</sub>O, and added to the reversed-phase column diphenyl 219TP5415 (Vydac), and elution was carried out on a gradient of 22%-25% CH<sub>3</sub>CN. As a result, the activity to specifically promote release of arachidonic acid metabolites from CHO-19P2 cells was converged by recovered in one elution peak obtained with 23% CH<sub>3</sub>CN [Fig. 19]. The peak activity fraction from the reverse-phased column diphenyl 219TP5415 was lyophilized, dissolved with DMSO, suspended in 0.1% TFA/H<sub>2</sub>O, and added to the reversed-phase column  $\mu$ RPC C2/C18 SC 2.1/10 (Pharmacia), and elution was carried out on a gradient of 22%-23.5% CH<sub>3</sub>CN. As a result, the activity to specifically promote release of arachidonic acid metabolites from CHO-19P2 cells was detected in the two peaks eluted with 23.0% and 23.2% CH<sub>3</sub>CN [Fig. 20].

[Example 14]

- 30       Determination of the amino acid sequence of the peptide having the activity to specifically promote release of arachidonic acid metabolites from CHO-19P2 cells as purified from bovine hypothalamus
- 35       The amino acid sequence of the peptide (P-3)

having activity to specifically promote release of arachidonic acid metabolites from CHO-19P2 cells as purified in Example 13 was determined. The fraction of peak activity from the reversed-phase  $\mu$ RPC C2/C18 SC 2.1/10 was lyophilized and dissolved in 20  $\mu$ l of 70%  $\text{CH}_3\text{CN}$  and analyzed for amino acid sequence with the peptide sequencer (ABI.491). As a result, the sequence defined by SEQ ID NO:3 was obtained. However, the 7th and 19th amino acids were not determined by only the analysis of amino acid sequence.

[Example 15]

Preparation of the active substance (peptide) which specifically promotes release of arachidonic acid metabolites from CHO-19P2 cells as purified from bovine hypothalamus

Of the three active fractions obtained with Vydac C18 218TP5415 in Example 13, the active fraction (P-2) eluted with 23.0%  $\text{CH}_3\text{CN}$  was further purified. This active fraction was lyophilized, dissolved with DMSO, suspended in 0.1% TFA/ $\text{dH}_2\text{O}$ , and added to reversed-phase column diphenyl 219TP5415 (Vydac), and elution was carried out on a gradient of 21.0%-24.0%  $\text{CH}_3\text{CN}$ . As a result, activity to specifically promote release of arachidonic acid metabolites from CHO-19P2 cells was detected in a peak eluted with 21.9%  $\text{CH}_3\text{CN}$ . This fraction was lyophilized, dissolved with DMSO, suspended in 0.1% TFA/ $\text{dH}_2\text{O}$ , and added to reversed-phase  $\mu$ RPC C2/C18 SC 2.1/10 (Pharmacia), and elution was carried out on a  $\text{CH}_3\text{CN}$  gradient of 21.5%-23.0%. As a result, the activity to specifically promote release of arachidonic acid metabolites from CHO-19P2 cells converged in one peak eluted with 22.0%  $\text{CH}_3\text{CN}$  [Fig. 21].

[Example 16]

Determination of the amino acid sequence of the

peptide (P-2) purified from bovine hypothalamus which specifically promotes release of arachidonic acid metabolites from CHO-19P2 cells

The amino acid sequence of the peptide (P-2) having the activity to specifically promote release of arachidonic acid metabolites from CHO-19P2 cells as purified in Example 15 was determined. The peak activity fraction from the reversed-phase column  $\mu$ RPC C2/C18 SC 2.1/10 was lyophilized, dissolved in 20  $\mu$ l of 70%  $\text{CH}_3\text{CN}$ , and analyzed for amino acid sequence with the peptide sequencer (ABI, 492) (SEQ ID NO:4).  
[Example 17]

Preparation of a poly(A)<sup>+</sup>RNA fraction from bovine hypothalamus and synthesis of a cDNA  
Using Isogen (Nippon Gene), total RNA was prepared from one animal equivalent of bovine hypothalamus. Then, using Fast Track (Invitrogen), a poly(A)<sup>+</sup>RNA fraction was prepared. From 1  $\mu$ g of this poly(A)<sup>+</sup>RNA fraction, cDNA was synthesized using 3' RACE system (GIBCO BRL) and Marathon cDNA amplification kit (Clontech) according to the manuals and dissolved in 20 and 10  $\mu$ l, respectively.  
[Example 18]

Acquisition of cDNA coding for the amino acid sequence established in Example 14

To obtain a cDNA coding for a polypeptide comprising the amino acid sequence established in Example 14, the acquisition of a base sequence coding for SEQ ID NO:1 was attempted in the first place. Thus, primers P5-1 (SEQ ID NO:35), P3-1 (SEQ ID NO:36), and P3-2 (SEQ ID NO:37) were synthesized. (In the Sequence Table, I represents inosine). Using 0.5  $\mu$ l of the cDNA prepared by 3' RACE in Example 17 as a template and EXTaq (Takara Shuzo Co., Japan) as DNA polymerase, 2.5  $\mu$ l of accompanying buffer, 200  $\mu$ M of accompanying dNTP, and primers P5-1 and P3-1 were added

each at a final concentration of 200 nM, with water added to make 25  $\mu$ l, and after one minute at 94°C, the cycle of 98°C x 10 seconds, 50°C x 30 seconds, 68°C x 10 seconds was repeated 30 times. This reaction mixture was diluted 50-fold with tricine-EDTA buffer and using 2.5  $\mu$ l of the dilution as a template and the primer combination of P5-1 and P3-2, the reaction was carried out in otherwise the same manner as described above. As the thermal cycler, Gene Amp 9600 (Perkin Elmer) was used. The amplification product was subjected to 4% agarose electrophoresis and ethidium bromide staining and a band of about 70 bp was cut out and subjected to thermal fusion, phenol extraction, and ethanol precipitation. The recovered DNA was subcloned into plasmid vector PCR<sup>TM</sup> II according to the manual of TA Cloning kit (Invitrogen). The vector was then introduced into *E. coli* JM109 and the resultant transformant was cultured in ampicillin-containing LB medium. The plasmid obtained with an automatic plasmid extractor (Kurabo) was reacted according to the manual of Dye Terminator Cycle Sequencing Kit (ABI) and decoded with a fluorescent automatic DNA sequencer (ABI). As a result, the sequence shown in Fig. 22 was obtained and confirmed to be part of the base sequence coding for SEQ ID NO:1.

[Example 19]

Acquisition of a bioactive polypeptide cDNA by RACE using the sequence established in Example 18

First, for amplification (5' RACE) of the sequence at 5' end, the two primers PE (SEQ ID NO:38) and PDN (SEQ ID NO:39) were synthesized by utilizing the sequence shown in Fig. 22. The cDNA prepared using Marathon cDNA amplification kit in Example 17 was diluted 100-fold with tricine-EDTA buffer. Then, in the same manner as Example 2, a reaction mixture was prepared using 2.5  $\mu$ l of the dilution and a combination

of the adapter primer AP1 accompanying the kit and the primer PE and after one minute at 94°C, the cycle of 98°C x 10 seconds and 68°C x 5 minutes was repeated 30 times. This reaction system was further diluted 50-  
5 fold with tricine-EDTA buffer and using 2.5 µl of the dilution as a template and the changed primer combination of AP1 and PDN, the reaction was conducted at 94°C for one minute, followed by 4 cycles of 94°C x 1 minute, 98°C x 10 seconds, 72°C x 5 minutes, 4 cycles  
10 of 98°C x 10 seconds, 70°C x 5 minutes, and 26 cycles of 98°C x 10 seconds, 68°C x 5 minutes. The amplification product was electrophoresed on 1.2% agarose gel and stained with ethidium bromide and a band of about 150 bp was cut out and centrifugally  
15 filtered through a centrifugal filter tube (Millipore), extracted with phenol, and precipitated from ethanol. The recovered DNA was subcloned into plasmid vector PCR<sup>TM</sup>II according to the manual of TA Cloning Kit (Invitrogen). The vector was then introduced into E.  
20 coli JM109 and the resulting transformant was cultured and the sequence of the inserted cDNA fragment was analyzed as in Example 18. As a result, the sequence shown in Fig. 23 was obtained. Based on this sequence, primers FB (SEQ ID NO:40) and FG (SEQ ID NO:41) were  
25 synthesized and the 3' sequence was cloned (3' RACE). Using the same template as that for 5' RACE in the same quantity and the combination of the accompanying adapter primer AP1 with the primer FC, PCR was carried out at 94°C for 1 minute, followed by 5 cycles of 98°C  
30 x 10 seconds, 72°C x 5 minutes, 5 cycles of 98°C x 10 seconds, 70°C x 5 minutes, and 25 cycles of 98°C x 10 seconds, 68°C x 5 minutes. Then, using 2.5 µl of a 50-fold dilution of this reaction mixture in tricine-EDTA buffer as the template and the combination of the  
35 accompanying primer AP2 with the primer FB, the reaction was further conducted at 94°C for one minute,

followed by 4 cycles of 98°C x 10 seconds, 72°C x 5 minutes, 4 cycles of 98°C x 10 seconds, 70°C x 5 minutes, and 27 cycles of 98°C x 10 seconds, 68°C x 5 minutes. The amplification product was electrophoresed on 1.2% agarose gel and stained with ethidium bromide and a band of about 400 bp was cut out and the DNA was recovered as in 5'-RACE. This DNA fragment was subcloned into plasmid vector pCR<sup>TM</sup>II and introduced into *E. coli* JM109 and the sequence of the inserted cDNA fragment in the resulting transformant was analyzed. From the results of 5' RACE and 3' RACE, the DNA sequence [Fig. 24] coding for the complete coding region of the bioactive polypeptide defined by SEQ ID NO:1 was established. Thus, in Fig. 24 (a) and (b), the base<sup>134</sup> is G, the base<sup>184</sup> is T or C, and the base<sup>245</sup> was T or C.

The cDNA shown in Fig. 24 was the cDNA encoding a polypeptide consisting of 98 amino acids. The fact that the amino acids in 1 - 22-positions comprise a cluster of hydrophobic amino acids taken together with the fact that the N-terminal region of the active peptide begins with Ser in 23-position as shown in Example 14 suggested that the amino acids 1-22 represent a secretion signal sequence. On the other hand, the Gly Arg Arg Arg sequence in 54-57 positions of the polypeptide was found to be a typical amino acid sequence motif which exists in the event of cleavage of a bioactive peptide. As it is the case with this cleavage motif, it is known that because of the presence of Gly, the C-terminus of the product peptide is frequently amidated.

The P-3 N-terminal sequence data of Example 14 and P-2 N-terminal sequence data in Example 16 coupled with this GlyArgArgArg sequence suggest that at least some of the bioactive peptides cut out from the polypeptide encoded by this cDNA are defined by SEQ ID NO:3, SEQ ID



NO:4, SEQ ID NO:5, SEQ ID NO:6, SEQ ID NO:7, SEQ ID NO:8, SEQ ID NO:9 or SEQ ID NO:10.

[Example 20]

5 Acquisition of a DNA fragment comprising the full coding region of bovine-derived bioactive polypeptide cDNA by PCR

Using the cDNA prepared with Marathon cDNA amplification kit in Example 17 as a template, a DNA fragment including the entire coding region of bioactive polypeptide cDNA was constructed. First, based on the sequence of cDNA elucidated in Example 19, two primers having base sequences defined by SEQ ID NO:42 and SEQ ID NO:43, respectively, were synthesized. BOVF

15 5'-GTGTCGACGAATGAAGGCGGTGGGGGCCTGGC-3' (SEQ ID NO:42)  
BOVR (24 mer)

5'-AGGCTCCCGCTGTTATTCCTGGAC-3' (SEQ ID NO:43)

BOVF contains the initiation codon of bioactive polypeptide cDNA and is a sense sequence corresponding to -2 - +22 (A of the initiation codon ATG being reckoned as +1) with restriction enzyme SalI site added. On the other hand, BOVR is an antisense sequence corresponding to +285 - +309 which includes the termination codon of bioactive polypeptide cDNA.

25 The PCR was conducted as follows. The cDNA prepared using Marathon cDNA amplification kit in Example 17 was diluted 100-fold in Tris-HCl-EDTA buffer and using 2.5 µl of the dilution, a reaction mixture was prepared as in Example 2 and subjected to 94°C x 1 minute, 3 cycles of 98°C x 10 seconds, 72°C x 5 minutes, 3 cycles of 98°C x 10 seconds, 70°C x 5 minutes, and 27 cycles of 98°C x 10 seconds, 68°C x 5 minutes. The amplification product was subjected to 2% agarose electrophoresis and ethidium bromide staining and a band of about 320 bp was cut out. The DNA was recovered and subcloned in plasmid vector pCR<sup>TM</sup>II as in

Example 3. The vector was introduced into Escherichia coli JM109 to provide the transformant E. coli JM109/pBOV3. The sequence of the cDNA fragment inserted in the transformant was then analyzed. As a result, this DNA fragment was confirmed to be a fragment covering the entire coding region of the bioactive polypeptide cDNA.

[Example 21]

Synthesis of Ser-Arg-Ala-His-Gln-His-Ser-Met-Glu-Ile-Arg-Thr-Pro-Asp-Ile-Asn-Pro-Ala-Trp-Tyr-Ala-Gly-Arg-Gly-Ile-Arg-Pro-Val-Gly-Arg-Phe-NH<sub>2</sub> (19P2-L31)

1) Synthesis of Ser(Bzl)-Arg(Tos)-Ala-His(Bom)-Gln-His(Bom)-Ser(Bzl)-Met-Glu(OcHex)-Ile-Arg(Tos)-Thr(Bzl)-Pro-Asp(OcHex)-Ile-Asn-Pro-Ala-Trp(CHO)-Tyr(Br-Z)-Ala-Gly-Arg(Tos)-Gly-Ile-Arg(Tos)-Pro-Val-Gly-Arg(Tos)-Phe-PMBHA-resin

The reactor of a peptide synthesizer (Applied Biosystems 430A) was charged with 0.71 g (0.5 mmole) of commercial p-methyl-BHA resin (Applied Biosystems, currently Perkin Elmer). After wetting with DCM, the initial amino acid Boc-Phe was activated by the HOBt/DCC method and introduced into the p-methyl-BHA resin. The resin was treated with 50% TFA/DCM to remove Boc and make the amino group free and neutralized with DIEA. To this amino group was condensed the next amino acid Boc-Arg(Tos) by the HOBt/DCC method. After the absence of unreacted amino function was verified by ninhydrin test, a sequential condensation of Boc-Gly, Boc-Val, Boc-Pro, Boc-Arg(Tos), Boc-Ile, Boc-Gly, Boc-Arg(Tos), Boc-Gly, Boc-Ala, Boc-Tyr(Br-Z) was carried out. The Boc-Ala, Boc-Tyr(Br-Z), the condensation of which was found insufficient by ninhydrin test, was recondensed to complete the reaction. The resin was dried and a half of the resin was withdrawn. To the remainder, Boc-

Trp(CHO), Boc-Ala, Boc-Pro, Boc-Asn, Boc-Ile, Boc-Asp(OcHex), Boc-Pro, Boc-Thr(Bzl), Boc-Arg(Tos), Boc-Ile, Boc-Glu(OcHex), Boc-Met, Boc-Ser(Bzl), Boc-His(Bom), Boc-Gln, Boc-His(Bom), Boc-Ala, Boc-Arg(Tos),  
 5 Boc-Ser(Bzl) were serially condensed and recondensed until sufficient condensation was confirmed by ninhydrin test. After introduction of the full sequence of amino acids of 19P2-L31, the resin was  
 10 treated with 50% TFA/DCM to remove Boc groups on the resin and, then, dried to provide 1.28 g of the peptide resin.

2) Synthesis of Ser-Arg-Ala-His-Gln-His-Ser-Met-Glu-Ile-Arg-Thr-Pro-Asp-Ile-Asn-Pro-Ala-Trp-Tyr-Ala-Gly-Arg-Gly-Ile-Arg-Pro-Val-Gly-Arg-Phe-NH<sub>2</sub>(19P2-L31) seq. 131-141

15 In a Teflon hydrogen fluoride reactor, the resin obtained in 1) was reacted with 3.8 g of p-cresol, 1 ml of 1,4-butanedithiol, and 10 ml of hydrogen fluoride at 0°C for 60 minutes. The hydrogen fluoride and 1,4-butanedithiol (1 ml) were distilled off under reduced  
 20 pressure and the residue was diluted with 100 ml of diethyl ether, stirred, filtered through a glass filter, and the fraction on the filter was dried. This fraction was suspended in 50 ml of 50% acetic acid/H<sub>2</sub>O and stirred to extract the peptide. After separation  
 25 of the resin, the extract was concentrated under reduced pressure to about 5 ml and chromatographed on Sephadex G-25 (2 x 90 cm). Development was carried out with 50% acetic acid/H<sub>2</sub>O and the 114 ml - 181 ml  
 30 fraction was pooled and lyophilized to recover 290 mg of white powders containing 19P2-L31. The powders were applied to a reversed-phase column of LiChrorep RP-18 (Merck) and repeatedly purified by gradient elution using 0.1% TFA/H<sub>2</sub>O and 0.1% TFA-containing 30%  
 35 acetonitrile/H<sub>2</sub>O. The fraction eluted at about 25% acetonitrile was pooled and lyophilized to provide 71 mg of white powders.

Mass spectrum (M+H)<sup>+</sup> 3574.645

HPLC elution time 18.2 min.

Column conditions

Column: Wakosil 5C18 (4.6 x 100 mm)

5 Eluent: A (0.1% TFA/H<sub>2</sub>O)

B (0.1% TFA-containing 50 (%  
acetonitrile/H<sub>2</sub>O)

Linear gradient elution from A to B (25 min.)

Flow rate: 1.0 ml/min.

10 [Example 22]

Synthesis of Ser-Arg-Ala-His-Gln-His-Ser-Met(O)-Glu-  
Ile-Arg-Thr-Pro-Asp-Ile-Asn-Pro-Ala-Trp-Tyr-Ala-Gly-  
Arg-Gly-Ile-Arg-Pro-Val-Gly-Arg-Phe-NH<sub>2</sub>(19P2-L31(O))

15 In 20 ml of 5% acetic acid/H<sub>2</sub>O was dissolved 6 mg  
of synthetic 19P2-L31 and the Met only was selectively  
oxidized with 40 µl of 30% H<sub>2</sub>O<sub>2</sub>. After completion of  
the reaction, the reaction mixture was immediately  
applied to a reversed-phase column of LiChroprep RP-18  
(Merck) for purification to provide 5.8 mg of the  
20 objective peptide.

Mass spectrum (M+H)<sup>+</sup> 3590.531

HPLC elution time 17.9 min.

Column conditions

Column: Wakosil 5C18 (4.6 x 100 mm)

25 Eluent: A (0.1% TFA/H<sub>2</sub>O)

B (0.1% TFA-containing 50% aceto  
nitrile/H<sub>2</sub>O)

Linear gradient elution from A to B (25 min.)

Flow rate: 1.0 ml/min.

30 [Example 23]

Synthesis of Thr-Pro-Asp-Ile-Asn-Pro-Ala-Trp-Tyr-Ala-  
Gly-Arg-Gly-Ile-Arg-Pro-Val-Gly-Arg-Phe-NH<sub>2</sub>(19P2-L20)

35 To the resin subjected to condensations up to Boc-  
Tyr(Br-Z) in Example 21-1) was further condensed Boc-  
Trp(CHO), Boc-Ala, Boc-Pro, Boc-Asn, Boc-Ile, Boc-

Asp(OcHex), Boc-Pro, Boc-Thr(Bzl) serially in the same manner to provide 1.14 g of Boc-Thr(Bzl)-Pro-Asp(OcHex)-Ile-Asn-Pro-Ala-Trp(CHO)-Tyr(Br-Z)-Ala-Gly-Arg(Tos)-Gly-Ile-Arg(Tos)-Pro-Val-Gly-Arg(Tos)-Phe-pMBHA-resin. This resin was treated with hydrogen fluoride and columnwise purified in the same manner as Example 21-2) to provide 60 mg of white powders.

Mass spectrum (M+H)<sup>+</sup> 2242.149

HPLC elution time 10.4 min.

10 Column conditions

Column: Wakosil 5C18 (4.6 x 100 mm)

Eluent: A (0.1% TFA-containing 15% acetonitrile/H<sub>2</sub>O)

15 B (0.1% TFA-containing 45% acetonitrile/H<sub>2</sub>O)

Linear gradient elution from A to B (15/min.)

Flow rate: 1.0 ml/min.

[Example 24]

20 Determination of arachidonic acid metabolites-releasing activity of synthetic peptide (19P2-L31)

The activity of the peptide (19P2-L31) synthesized in Example 21 to specifically release arachidonic acid metabolites from CHO-19P2 cells was assayed in the same manner as Example 11. The synthetic peptide was dissolved in degassed dH<sub>2</sub>O at a concentration of 10<sup>-3</sup>M and diluted with 0.05% BSA-HBSS and the activity to promote release of arachidonic acid metabolites from CHO-19P2 cells at each concentration was assayed using the amount of [<sup>3</sup>H]arachidonic acid metabolites as the indicator. As a result, concentration-dependent arachidonic acid metabolite-releasing activity was detected over the range of 10<sup>-12</sup>M - 10<sup>-6</sup>M [Fig. 25]. When the arachidonic acid metabolite-releasing activity of peptide 19P2-L31(O), i.e. the methionine-oxidation product of 19P2-L31 synthesized in Example 22, was compared with that of 19P2-L31, it was found that the

activity of 19P2-L31(O) was equivalent to the activity of 19P2-L31 as can be seen from Fig. 26.

[Example 25]

Determination of arachidonic acid metabolites-releasing activity of synthetic peptide (19P2-L20)  
The activity of the synthetic equivalent (19P2-L20) of natural peptide P-2 as synthesized in Example 23 to specifically promote release of arachidonic acid metabolites from CHO-19P2 cells was determined as in Example 11. Thus, the synthetic peptide was dissolved in degassed dH<sub>2</sub>O at a final concentration of 10<sup>-3</sup>M and this solution was serially diluted with 0.05% BAS-HBSS. The activity to specifically promote release of arachidonic acid metabolites from CHO-19P2 cells at each concentration was assayed using the amount of [<sup>3</sup>H]arachidonic acid metabolites as the indicator.

As a result, concentration-dependent arachidonic acid metabolite-releasing activity was detected over the range of 10<sup>-12</sup> - 10<sup>-6</sup>M in nearly the same degree as 19P2-L31 [Fig. 27].

[Example 26]

Analysis of the coding region base sequence of bovine genomic DNA  
pBOV3 was digested with restriction enzyme EcoRI and after fractionation by agarose gel electrophoresis, the DNA corresponding to the cDNA fragment was recovered to prepare a probe. This DNA was labeled with <sup>32</sup>P using a multiprime DNA labeling kit (Amersham). About 2.0x10<sup>6</sup> phages of Bovine Genomic Library (Clontech BL1015j) constructed using cloning vector EMBL3 SP6/T7 and Escherichia coli K802 as the host were seeded in an LB agar plate and cultured overnight for plaque formation. The plaques were transferred to a nitrocellulose filter and after alkaline modification and neutralization, heat-treated (80°C, 2 hours) to inactivate the DNA. This filter was

incubated with the labeled probe in 50% formamide-Hybri  
buffer (50% formamide, 5 x Denhardt solution, 4 x SSPE,  
0.1 mg/ml heat-denatured salmon sperm DNA, 0.1% SDS) at  
42°C overnight for hybridization. After this  
5 hybridization, the filter was washed with 2 x SSC, 0.1%  
SDS at room temperature for 1.5 hours, and further  
washed in the same buffer at 55°C for 30 minutes.  
Detection of the clone hybridizing with the probe was  
carried out on Kodak X-ray film (X-OMAT<sup>TM</sup>AR) after 4  
10 days of exposure using a sensitization screen at -80°C.  
After development of the film, the film was collated  
with plate positions and the phages which had  
hybridized were recovered. Then, plating and  
hybridization were repeated in the same manner for  
15 cloning of the phages.

The cloned phages were prepared on a large scale  
by the plate lysate method and the phage DNA was  
extracted. Then, cleavage at the restriction enzyme  
SalI and BamHI cleavage sites at both ends of the  
20 cloning site of the vector and detection of the  
inserted fragment derived from bovine genomic DNA was  
carried out by 1.2% agarose gel electrophoresis [Fig.  
28]. As a result, in the case of BamHI digestion, 3  
fragments were detected in addition to the bands  
25 derived from the phages. In the case of SalI  
digestion, one band overlapping the phage band was  
detected. The SalI-digested fragment being considered  
to harbor the full length and in order to subclone this  
fragment into a plasmid vector, it was ligated to BAP  
30 (E. coli-derived alkaline phosphatase)-treated plasmid  
vector pUC18 (Pharmacia) and introduced into E. coli  
JM109. From this microorganism, a genome-derived SalI  
fragment-inserted plasmid DNA was prepared on a  
production scale and the base sequence in the  
35 neighborhood of its coding region was analyzed using  
Perkin Elmer Applied Biosystems 370A fluorescent

5

10

15

20

25

30

35



of the primers, ExTaq (Takara Shuzo Co., Japan) as DNA polymerase, and 2.5 µl of the accompanying buffer, with a sufficient amount of water to make a total of 25 µl. The reaction was carried out at 94°C for 1 minute, followed by 40 cycles of 98°C x 10 seconds, 50°C x 30 seconds, and 72°C x 5 seconds, and the reaction mixture was then allowed to stand at 72°C for 20 seconds. The thermal cycler used was GeneAmp2400 (Perkin Elmer). The amplification product was subjected to 4% agarose electrophoresis and ethidium bromide staining and the band of about 80 bp was cut out. Then, in the manner described in Example 19, the DNA was recovered, subcloned into plasmid vector pCR<sup>TM</sup>II, and introduced into *E. coli* JM109, and the inserted cDNA fragment was sequenced. As a result, a partial sequence of rat bioactive polypeptide could be obtained. Based on this sequence, two primers, namely RA (SEQ ID NO:75) for 3' RACE and RC (SEQ ID NO:76) for 5' RACE were synthesized and 5' and 3' RACEs were carried out.

RA:5'-CARCAYTCCATGGAGACAAGAACCCC-3'  
(where R means A or G; Y means T or G) (SEQ ID NO:75)  
RC:5'-TACCAGGCAGGATTGATACAGGGG-3'

(SEQ ID NO:76)

As a template, the template synthesized using Marathon cDNA amplification kit (Clontech) in Example 27 was diluted 40-fold with the accompanying tricine-EDTA buffer and 2.5 µl of the dilution was used. As primers, RA and the adapter primer AP1 accompanying the kit were used for 3' RACE, and RC and AP1 for 5' RACE. The reaction mixture was prepared in otherwise the same manner as above. The reaction conditions were 94°C x 1 minute, 5 cycles of 98°C x 10 seconds, 72°C x 45 seconds, 3 cycles of 98°C x 10 seconds, 70°C x 45 seconds, and 40 cycles of 98°C x 10 seconds, 68°C x 45 seconds. As a result, a band of about 400 bp was obtained from 3' RACE and bands of about 400 bp and 250

bp from 5' RACE. These bands were recovered in the same manner as above and using them as templates and the primers used in the reaction, sequencing was carried out with Dye Terminator Cycle Sequencing Kit (ABI). As a result, the sequence up to poly A could be obtained from the region considered to be the 5' noncoding region.

[Example 29]

Acquisition of the full-length cDNA of rat bioactive polypeptide by PCR

Based on the sequence obtained in Example 28, two primers, viz. rF for the region including the initiation codon (SEQ ID NO:77) and rR for the 3' side from the termination codon (SEQ ID NO:78), were synthesized to amplify the fragment including the full-length cDNA.

rF:5'-GGCATCATCCAGGAAGACGGAGCAT-3' (SEQ ID NO:77)

rR:5'-AGCAGAGGAGAGGGAGGGTAGAGGA-3' (SEQ ID NO:78)

Using the cDNA prepared using Moloney mouse leukemia reverse transcriptase in Example 27 as a template and ExTaq (Takara Shuzo Co., Japan), PCR was carried out by repeating 40 cycles of 95°C x 30 seconds, 68°C x 60 seconds. The amplification product was subjected to agarose electrophoresis and ethidium bromide staining and a band of about 350 bp was cut out. The DNA was recovered, subcloned into plasmid vector pCR<sup>TM</sup>II, and introduced into E. coli JM109 as in Example 19. The plasmid was extracted from the transformant and the base sequence was determined. As a result, E. coli JM 109/pRAV3 having the full-length cDNA of rat bioactive polypeptide was obtained [Fig. 32].

[Example 30]

Synthesis of cDNA from the human total brain poly(A)<sup>+</sup>RNA fraction

From 1 µg of human total brain poly(A)<sup>+</sup>RNA

fraction (Clontech), cDNA was synthesized with Marathon cDNA amplification kit (Clontech) according to the manual and dissolved in 10  $\mu$ l. In addition, the random DNA hexamer (BRL) was added as primer to 5  $\mu$ g of the same poly(A)<sup>+</sup>RNA fraction and using Moloney mouse leukemia reverse transcriptase (BRL) and the accompanying buffer, complementary DNA was synthesized. The reaction product was precipitated from ethanol and dissolved in 30  $\mu$ l of TE.

10 [Example 31]

Acquisition of human bioactive polypeptide cDNA by RACE

From the amino acid sequence of rat bioactive polypeptide established in Example 28 [Fig. 33], the well-preserved regions of rat and bovine polypeptides were selected and the following 3 primers R1, (SEQ ID NO:79), R3 (SEQ ID NO:80), and R4 (SEQ ID NO:81) were synthesized. Then, amplification of the region flanked by them was attempted by PCR using human cDNA as a template. Referring to Fig. 33, bovine. aa represents the amino acid sequence of bovine polypeptide, bovine. seq represents the base sequence of the DNA coding for bovine polypeptide, and rat. seq represents the base sequence of the DNA coding for rat polypeptide.

25 R1:5'-ACGTGGCTTCTGTGCTTGCTGC-3' (SEQ ID NO:79)  
 R3:5'-GCCTGATCCCGCGGCGCCGTGTACCA-3' (SEQ ID NO:80)  
 R4:5'-TTGCCCTTCTCCTGCCGAAGCGGCCC-3' (SEQ ID NO:81)

The cDNA prepared using Marathon cDNA amplification kit (Clontech) in Example 30 was diluted 30-fold with tricine-EDTA buffer and 0.25  $\mu$ l of the dilution was used as a template. The reaction mixture was composed of 200  $\mu$ M of dNTP, 0.2  $\mu$ M each of the primers R1 and R4, a 50:50 mixture of Taq Start Antibody (Clontech) and DNA polymerase ExTaq (Takara Shuzo Co., Japan), 2.5  $\mu$ l of the accompanying buffer, and a sufficient amount of water to make a total of 25

1      $\mu$ l. The reaction conditions were 94°C x 1 minute,  
 followed by 42 cycles of 98°C x 10 seconds, 68°C x 40  
 seconds, and 1 minute of standing at 72°C. Then, using  
 1  $\mu$ l of a 100-fold dilution of the above reaction  
 5     mixture in tricine-EDTA buffer as a template, the same  
 reaction mixture as above except that the primer  
 combination was changed to R1 and R3 was prepared and  
 PCR was carried out in the sequence of 94°C x 1 minute  
 and 25 cycles of 98°C x 10 seconds, 68°C x 40 seconds.  
 10     The amplification product was subjected to 4% agarose  
 electrophoresis and ethidium bromide staining. As a  
 result, a band of about 130 bp was obtained as  
 expected. This band was recovered in the same manner  
 as in Example 28 and using the recovered fragment as a  
 15     template, sequencing was carried out with Dye  
 Terminator Cycle Sequencing Kit (ABI). As a result, a  
 partial sequence of human bioactive polypeptide could  
 be obtained. Therefore, based on this sequence,  
 primers HA (SEQ ID NO:82) and HB (SEQ ID NO:83) were  
 20     synthesized for 3' RACE and primers HE (SEQ ID NO:84)  
 and HF (SEQ ID NO:85) for 5' RACE and 5' and 3' RACEs  
 were carried out.

HA:5'-GGCGGGGGCTGCAAGTCGTACCCATCG-3' (SEQ ID NO:82)

HB:5'-CGGCACTCCATGGAGATCCGCACCCCT-3' (SEQ ID NO:83)

25     HE:5'-CAGGCAGGATTGATGTCAGGGGTGCGG-3' (SEQ ID NO:84)

HF:5'-CATGGAGTGCCGATGGGTACGACTTGC-3' (SEQ ID NO:85)

As the template, 2.5  $\mu$ l of a 20-fold dilution of  
 the cDNA prepared in Example 30 in tricine-EDTA buffer  
 was used. For the initial PCR, reaction mixtures were  
 30     prepared in the same manner as above except that HA and  
 adapter primer AP1 were used for 3' RACE and HE and AP1  
 for 5' RACE. The reaction sequence was 94°C x 1  
 minute, 5 cycles of 98°C x 10 seconds, 72°C for 35  
 seconds, 5 cycles of 98°C x 10 seconds, 70°C x 35  
 35     seconds, and 40 cycles of 98°C x 10 seconds, 68°C x 35  
 seconds. Then, using 1  $\mu$ l of a 100-fold dilution of

this reaction mixture in tricine-EDTA buffer as a template, a second PCR was carried out in the same cycles as the first PCR. However, the reaction mixture was prepared using primers HB and AP1 for 3' RACE or HF and AP2 for 5' RACE and Klen Taq (Clontech) as DNA polymerase and the accompanying buffer. As a result, a band of about 250 bp was obtained from 3' RACE and a band of about 150 bp from 5'-RACE. These bands were sequenced by the same procedure as above and using them in combination with the partial sequence obtained previously, the sequence from the region presumed to be 5'-noncoding region to polyA of human bioactive polypeptide was obtained.

[Example 32]

Acquisition of human bioactive polypeptide full-length cDNA by PCR

Based on the sequence obtained in Example 31, two primers 5H (SEQ ID NO:86) and 3HN (SEQ ID NO:87) were synthesized for amplification of a fragment including full-length cDNA.

5H: 5'-GGCCTCCTCGGAGGAGCCAAGGGATGA-3' (SEQ ID NO:86)

3HN: 5'-GGGAAAGGAGCCCGAAGGAGAGGAGAG-3' (SEQ ID NO:87)

Using 2.5  $\mu$ l of the cDNA prepared using Moloney mouse leukemia reverse transcriptase (BRL) in Example 30 as a template and the reaction mixture prepared using Klen Taq DNA polymerase (Clontech), the PCR reaction was conducted in the sequence of 94°C x 1 minute and 40 cycles of 98°C x 10 seconds, 68°C x 30 seconds. The fragment of about 360 bp obtained was recovered and subcloned (pCR<sup>TM</sup> 2.1 was used as the vector) in otherwise the same manner as Example 29. The plasmid was recovered and its base sequence was determined. As a result, *E. coli* JM109/pHOV7 harboring the human bioactive polypeptide full-length cDNA was obtained [Fig. 34]. In regard to the amino acid sequence of the translation region, a comparison was

made between this human bioactive polypeptide and the bovine polypeptide shown in Example 20 or the rat polypeptide in Example 29 [Fig. 35].

[Example 33]

5           An orphan G-protein coupled receptor, UHR-1, has been cloned from rat hypothalamic suprachiasmatic nuclei, and its nucleotide sequences have been reported (Biochemical and Biophysical Research Communications, vol. 209, No.2, pp606-613, 1995., Genbank Accession  
10       Number: S77867). A protein coded by UHR-1 showed 91.6% identity over 359 amino acids with that of phGR3, suggesting UHR-1 is a counterpart of hGR3. To confirm this we cloned a cDNA for UHR-1 coding regions and established a CHO cells stably expressing UHR-1 as  
15       described below. Poly(A)<sup>+</sup> RNA was prepared from rat anterior pituitary using a FastTrack<sup>TM</sup> Kit (Invitrogen Co.), and cDNA was synthesized from 0.2 µg of this with Takara RNA PCR Kit (Takara). The cDNA was dissolved in 10 µl of distilled water, and used as a template for  
20       the following PCR. To isolate UHR-1 cDNA, two primers, namely 5'-GTTACACAG(GTCGAC)ATGACCTCAC-3' [SEQ ID NO:95] (UHF), and 5'-CTCAGA(GCTAGC)AGAGTGTCATCAG-3' [SEQ ID NO:96] (UHR), were synthesized on the basis of the sequence of UHR-1 submitted to Genbank (Accession  
25       Number: S77867). In these primers, GTCGAC and GCTAGC indicate the SalI and NheI site respectively. Ex Taq (Takara) was admixed with an equal amount of Taq Start Antibody (Clontech Laboratories, Inc.) to prevent amplification of nonspecific products and primer  
30       dimers. Reaction mixture was prepared by adding 5 µl of the buffer attached to Ex Taq, 4 µl of dNTPs, 1 µl of the mixed solution of Ex Taq and Taq Start Antibody, and 1 µl of 50 µM each primers. The cDNA was diluted to one fifth with distilled water, and an aliquot (5  
35       µl) was added to the reaction mixture. PCR conditions were as follows: denatured at 95°C for 2 minutes,

followed by 27 cycles at 95°C for 30 seconds, 65°C for 30 seconds and 72°C for 1 minutes, and after these cycles at 72°C for 7 minutes.

The PCR products were separated with 1.2% agarose gel and stained with ethidium bromide. Slices of agarose gel containing the band about 1.1 kbp were cut out with razor blade, and then filtered using an Ultra Free filter unit (Millipore). The eluent was extracted with phenol: chloroform and precipitated in ethanol.

The amplified DNA was subcloned into pCR<sup>TM</sup>II with a TA cloning Kit (Invitrogen Co.), and then introduced into *E. coli* JM109 competent cells. Transformants were selected in LB (Luria-Bertani) agar culture medium containing ampicillin, IPTG (isopropylthio-beta-D-galactoside), and X-gal (5-bromo-4-chloro-3-indolyl-beta-D-galactoside). The individual clones were cultured in an LB culture medium containing ampicillin and treated with an automatic plasmid extracting machine (Kurabo) to prepare plasmid DNAs respectively.

Sequencing was carried out with a ABI PRISM Dye Terminator Cycle Sequencing Kit FS (Perkin-Elmer), and an ABI automatic sequencer. In the Fig. 52, underlines indicate the sequences corresponding to the parts of primer sequences. Double-lined bases indicate the base substitution compared with the sequence data reported, and one of these substitutions was accompanied by an amino acid substitution from <sup>289</sup>Leu(CTC) to <sup>289</sup>Val(GTC). A plasmid, pCRII-UHR-1, containing the UHR-1 cDNA fragment was thus constructed.

UHR-1 cDNA expression plasmid was prepared as follows. First, pCRII-UHR-1 was digested with NheI and SalI. The resultant fragment of about 1.1 kbp was separated through electrophoresis using a 1.2% agarose gel and precipitated as above. The DNA fragment was then ligated into the NheI-SalI site of pAKKO-111H, with a Ligation System (Takara). A resultant

expression plasmid, pAKKO-UHR-1 was introduced into E. coli JM109.

CHO dhfr<sup>-</sup> cells were grown in 10 cm diameter Petri dishes at the cell number of  $1 \times 10^6$ , and cultured at 37°C for 24 hours in  $\alpha$ -MEM containing 10% of fetal bovine serum. The expression plasmid (20  $\mu$ g) was introduced into the cells by a liposome method using a Gene Transfer (Nippon Gene). After 24 hours from the introduction, the medium was substituted with fresh one. After additional 24 hour incubation, the culture medium was changed to a Selection medium,  $\alpha$ -MEM without nucleosides containing 10% of dialyzed fetal bovine serum. Culture was carried out until cells growing in the Selection medium were obtained. CHO-UHR-1 which highly expressed UHR-1 was thus established.

[Example 34]

Radioiodination of 19P2-L31 and receptor binding experiments

19P2-L31 was radioiodinated with [ $^{125}$ I]-Bolton-Hunter Reagent (NEN.Dupont; NEX-120) as follows. Two hundred microliter of [ $^{125}$ I]-Bolton-Hunter Reagent was dried in a 500  $\mu$ l Eppendorf tube with N<sub>2</sub> gas. The dried reagent was dissolved in 2  $\mu$ l of acetonitrile, and then mixed with 4 ml of 50 mM phosphate buffer (pH 8.0) and 4  $\mu$ l of 19P2-L31  $3 \times 10^{-4}$ M. The mixture was incubated at room temperature for 40 min and the reaction was stopped by adding 5  $\mu$ l of 1.0 M glycine. The all reaction mixture was diluted with 300  $\mu$ l of 18% acetonitrile and injected onto reverse-phase HPLC column TSK gel ODS-80TM (4.6x100mm; TOSO). The radioiodinated 19P2-L31 was eluted with a linear gradient of acetonitrile concentration from 18 to 32.4% in 0.1% trifluoroacetic acid for 24 min at a flow rate of 1 ml/min. The peak fraction of radioiodinated 19P2-L31 was collected and diluted with twice volume of 50 mM Tris-HCl(pH7.5) containing 0.1% BSA and 0.05% CHAPS,



and then stored at  $-20^{\circ}\text{C}$ .

Receptor binding experiments were performed with  $[^{125}\text{I}]\text{-19P2-L31}$  as follows. As receptor-expressing CHO cells, CHO-19P2-9; mono-clone of CHO-19P2, CHO-UHR-1, and mock CHO were used in this experiment. CHO-19P2-9 cells are ones selected from CHO-19P2 cells by ultradilution technique using 96-well microplate as clone which indicated stronger arachidonic acid metabolic-release promoting reaction by 19P2-L31. The mock CHO cells are ones for control which were transformed with expression vector pAKKO alone. These cells cultured in flasks for culturing tissues were harvested with 5 mM EDTA/PBS, and then resuspended in HBSS containing 0.05% BSA and 0.05% CHAPS at  $0.5 \times 10^7$  cells/ml. The cell suspensions were incubated with 200 pM  $[^{125}\text{I}]\text{-19P2-L31}$  for 2.5 hr at room temperature in a 100  $\mu\text{l}$  total volume. The reaction mixture were diluted with 2 ml of an ice-cold beffer (50 mM Tris-HCl pH7.5 containing 5 mM EDTA, 0.05% BSA, and 0.05%CHAPS) and immediately filtered though glass filters GF/F (Whattman) which were pre-wetted with the buffer containing 0.3% polyethylenimine. The glass filters were subjected to  $\gamma$ -counting. Non-specific binding was determined in the presence of 200 nM unlabeled 19P2-L31.

[Fig. 36] shows receptor binding experiments with  $[^{125}\text{I}]\text{-19P2-L31}$  on live cells.

Specific binding of  $[^{125}\text{I}]\text{-19P2-L31}$  was detected on CHO cells which were expressed with hGR3 and rat homolog UHR-1 respectively. The experiments were performed in triplicate. These results show that the proteins encoded by hGR3 and UHR-1 is functioning as the specific receptor of 19P2-L31.

[Example 35]

Release of arachidonic acid metabolites from CHO-19P2-9 and CHO-UHR1 by 19P2-L31

Same as described in Example 11, the release activity of arachidonic acid metabolite was measured on CHO-19P2-9 and CHO-UHR1 and mock CHO.

[Fig. 37] shows the release activity of arachidonic acid metabolite on CHO-19P2-9 and CHO-UHR1 by 19P2-L31.

On CHO cells which were expressed with rat homolog UHR1, the release activity of arachidonic acid metabolite was detected same as CHO-19P2-9. The experiments were performed in duplicate. These results show that the protein encoded by UHR-1 is functioning as the specific receptor as well as hGR3.

[Example 36]

Quantification of rat 19P2 ligand and rat UHR-1 mRNA, BBRC, 209,606-613, 1995) by RT-PCR  
(1) Preparation of poly(A)+RNA and cDNA synthesis from rat tissues.

Poly(A)+RNA was isolated from a variety of tissues in rats (Wister strain, male, 8 weeks old) by homogenization with Isogen (Nippon Gene) followed by an oligo (dT)-cellulose chromatography (Pharmacia). One  $\mu$ g of poly(A)+RNA was treated with DNase I (Amplification grade, GibcoBRL) to eliminate the contamination of genomic DNA. DNase I was inactivated by the addition of 25 mM EDTA solution at 65°C. Then RNA (160 ng) was reverse-transcribed in 40  $\mu$ l of a reaction mixture containing 10 mM of Tris-HCl (pH 8.3), 2.5  $\mu$ M of random hexamers (Takara), 0.4 mM of each dNTP, and 10 U of AMV reverse transcriptase XL (Takara). The samples were incubated at 30°C for 10 min followed by 42°C for 1h, then 99°C for 5 min to stop the reaction. The reaction mixture was purified by ethanol precipitation, and then the cDNA was diluted to 40  $\mu$ l with tricine-EDTA buffer (correspond to 4 ng poly(A)+RNA/ $\mu$ l).

(2) Construction of positive control plasmid vectors

Rat glycerolaldehyde-3-phosphate-dehydrogenase (G3PDH) and rat UHR-1 cDNAs were isolated from rat pituitary tumor cell line GH<sub>3</sub> by means of RT-PCR. Poly(A)+RNA of GH<sub>3</sub> was prepared by FastTrack (Invitrogen), and cDNA was synthesized as Example 36(1). Oligonucleotide primers used for the amplification are as follows: rat G3PDH amplification primer set (Clontech), rRECF(5'-CCTGCTGGCCATTCTCCTGTCTTAC-3') (SEQ ID NO:88) and rRECR(5'-GGGTCCAGGTCCCGCAGAAGGTTGA-3') (SEQ ID NO:89) for UHR-1. The fragments amplified from GH<sub>3</sub> cDNA were subcloned with a TA cloning Kit (Invitrogen). The recombinant vectors were introduced into *E. coli* JM109. The transformant clones were cultured in a LB culture medium containing ampicillin, and the plasmid DNAs were prepared with a Quiagen Plasmid Midi Kit (Quiagen). The plasmid of rat ligand polypeptide was prepared from *E. coli* JM109/pRAV3 which was deposited.

### (3) Quantification RT-PCR

cDNA and plasmid DNA prepared in (1) and (2) above were diluted with distilled water to adequate concentrations and used as templates of quantitative RT-PCR. G3PDH, UHR-1, and ligand polypeptide cDNA fragments were amplified using human G3PDH amplifier (Clontech), rRECF and rRECR, and r19F(5'-GAAGACGGAGCATGGCCCTGAAGAC-3') (SEQ ID NO:91) and r19R(5'-GGCAGCTGAGTTGGCCAAGTCCAGT-3') (SEQ ID NO:91), respectively. Each reaction sample contained 100  $\mu$ M of dNTP mixture, 200 nM of each primer, 4  $\mu$ l of template DNA, 0.25  $\mu$ l of 50x KlenTaq DNA polymerase mix (Clontech), and 2.5  $\mu$ l of the buffer attached to KlenTaq DNA polymerase mix in a final volume of 25  $\mu$ l. PCR conditions for G3PDH were as follows: denatured at 94°C for 1 min, followed by 26 cycles at 98°C for 10 sec, at 65°C for 20 sec, and at 72°C for 40 sec. PCR conditions for UHR-1 and ligand polypeptide were as

follows: denatured at 94°C for 1 min, followed by 34 cycles at 98°C for 10 sec, and at 68°C for 25 sec. An aliquot 5 µl of each RT-PCR product was separated with 4% Nusieve 3:1 agarose gel (F.M.C.) electrophoresis and stained with ethidium bromide. The bands were quantified using a densitometry program (Advanced American Biotechnology).

The results measured the expression levels of UHR-1 and ligand polypeptide mRNA in the tissues were shown in Fig. 38 and 39 respectively. UHR-1 and ligand polypeptide mRNA were detected in all the tissues tested. The highest level of UHR-1 mRNA expression was detected in the pituitary, and moderate expression levels in the brain, whereas poorly expressed in peripheral tissues except for the adrenal glands. Ligand polypeptide mRNA expressed mainly in the hypothalamus and dorsal medulla among brain regions, and expressed comparatively high levels in the lung, thymus, pancreas, kidney, adrenal glands, and testis. These results show that the UHR-1 and ligand polypeptide play a significant role for the regulation of function in various tissues.

[Example 37]

Effect of 19P2-L31 on glucose-induced increase in plasma insulin concentration

Male Wistar rats (8-10w) were anesthetized by i.p. injection of pentobarbital (65 mg/kg). Glucose alone (86 mg/rat) or glucose and 19P2-L31 (675 pmol, 2.25 nmol, 6.75 nmol and 67.5 nmol/rat) were administered by bolus injection in the jugular vein. Blood samples were withdrawn from the contralateral vein. Plasma insulin concentration was determined with a radioimmunoassay kit (Amersham).

Administration of 19P2-L31 at the doses of 675 pmol, 2.25 nmol, and 6.75 nmol partially inhibited glucose-induced sharp increase (the first phase) in

plasma insulin concentration at 2 min postinjection and the blunt increase (the second phase) after 6 min postinjection. It completely inhibited the first and second phase of increase in insulin concentration at the dose of 67.5 nmol [Fig. 40].

[Example 38]

Effects of ligand polypeptide on motor activity of mouse

The effects of administration of 19P1-L31 to mouse lateral ventricle on motor activity were studied. The mature ICR male mice (weight at operation: about 35 g) were anesthetized by intraperitoneal administration of 50 mg/kg of pentobarbital, and then fixed on a stereotaxic apparatus. The skull of a said mouse was exposed, then a hole was made by dental drill for guide-cannulization into the left lateral ventricle. The tip of a stainless-steel guide-cannula (24G, length: 5 mm) for drug injection to lateral ventricle, was inserted to the position of AP: +0.6 mm (from bregma), L: left 1 mm and H: -1 mm (from dura matter). The guide-cannula was fixed onto the skull with adhesive. The cannula-implanted mice were housed as described above and were used for behavioral analysis at least 3 days after the operation.

Motor activity such as spontaneous motor activity and rearing was measured while each mouse was in a transparent acrylic cage (24 x 37 x 30 cm) within a soundproofed, illuminated (light up: at 6-18 o'clock) box. Tap water and laboratory chow were available ad libitum. Motor activity was measured by means of a Supermex (Muromachi Kikai). Drugs and PBS were administered at 2:30±30 p.m. At the administration, a stainless-steel micro-injection cannula (30G, length: 6 mm) was inserted into the guide-cannula. The micro-injection cannula was connected to a microsyringe pump with Teflon tube, and injection of PBS or a peptide

dissolved in PBS lasted for 2 minutes at a speed of 2  $\mu$ l/min. The micro-injection cannula was withdrawn after over a period of 2 minutes from end of injection, then motor activity was measured.

5       The results are expressed as a mean  $\pm$  S.E.M. Student's t test was used to determine the significance of differences between values from the mice treated with a peptide and the PBS-injected controls. For the purpose of this analysis,  $p < 0.05$  was assumed to be the  
10       minimal level of significance.

As shown in [Fig. 41], administration of 10 nmol of 19P2-L31 caused a significant increase in spontaneous motor activity at 70-105 min after injection. Rearing behavior also showed significant  
15       variation. While the administration of 1 nmol of 19P2-L31 did not cause statistically significant change of spontaneous motor activity, rearing behavior showed a significant decrease at only 105 min after injection [Fig. 42]. The administration of 0.1 nmol of 19P2-L31  
20       caused a significant increase at 25 min, 40 min and 70 min after injection. In that case, rearing behavior showed an increasing tendency similarly to spontaneous motor activity, however that was not statistically significant [Fig. 43]. The administration of 0.01 nmol  
25       of 19P2-L31 caused a significant increase at 20 min and 40 min after injection. In that case, rearing behavior showed an increasing tendency similarly to spontaneous motor activity, however that was not statistically significant [Fig. 44].

30       [Example 39]

Effects of ligand polypeptide on reserpine-induced hypothermia in mice

The mature ICR male mice (weight at operation: about 35 g) were anesthetized by administration of  
35       pentobarbital (50 mg/kg, i.p.), and then fixed on stereotaxic apparatus. The skull of a said mouse was

exposed, then a hole was made by dental drill for guide-cannulization into the left lateral ventricle. The tip of a stainless-steel guide-cannula (24G, length: 5 mm) for drug injection to lateral ventricle, was inserted to the position of AP: +0.6 mm (from bregma), L: left 1 mm and H: -1 mm (from dura matter). The guide-cannula was fixed onto the skull with adhesive. The cannula-implanted mice were housed as described above and were used for measurements of body temperature at least 3 days after the operation. Reserpine (Apoplon; Daiichi Pharmaceutical) was administered to mice at a dose of 3 mg/kg, s.c., and after 15 hours, each mouse was placed in a cage for the measurement. Then a stainless-steel micro-injection cannula (30G, length: 6 mm) was inserted into the guide-cannula. The micro-injection cannula was connected to a microsyringe pump with Teflon tube, and injection of PBS or a peptide dissolved in PBS lasted for 2 minutes at a speed of 1  $\mu$ l/min. The micro-injection cannula was withdrawn after over a period of 2 minutes from end of injection, then the temperature in rectum was measured.

The results are expressed as a mean  $\pm$  S.E.M. Student's t test was used to determine the significance of differences between values from the mice treated with a peptide and the PBS-injected controls. For the purpose of this analysis,  $p < 0.05$  was assumed to be the minimal level of significance.

As shown in [Fig. 45], body temperature which was lowered by reserpine increased significantly after a 10 nmol injection of 19P2-L31 in contrast to the control which PBS were administered. This increase of body temperature reached a maximum level at 45 min after administration of the peptide. On the other hand, there was no statistically significant difference in temperature variation between 1 nmol of 19P2-L31 and

the PBS-injected control throughout the experimental period.

[Example 40]

5           Effects of ligand polypeptide on blood pressure in rats

10           The inventors explored the influence of injection of 19P2-L31 into the area postrema of medula oblongata on blood pressure. Mature male Wistar rats (body weights at operation: ca 300 g) were anesthetized with pentobarbital 50 mg/kg i.p. and each animal was immobilized in a rat brain stereotaxic apparatus. The incisor bar was lowered by 3.3 mm from the interaural line. The skull was exposed, and using a dental drill a hole was made on the skull for implantation of a guide cannula. In addition, anchor screws were buried in two positions around the drilled hole. A stainless-steel guide cannula, AG-12 (0.4 mm inside dia., 0.5 mm out. dia., EICOM), was inserted in such a manner that its leading end would be situated in the upper part of the area postrema. For this purpose, the guide cannula was inserted from a forward direction at an angle of 20° with the perpendicular (Fig. 46; Note, however, that the drawing shows a microinjection cannula 1.0 mm longer than the guide cannula). With reference to the atlas of Paxinos and Watson (1986), the stereotaxic coordinates were AP: -6.0 mm (from interaural line), L: 0.0 mm, and H: +1.5 mm (from interaural line). The guide cannula was secured to the skull using an instant adhesive, a dental cement, and anchor pieces. A stainless-steel dummy cannula, AD-12 (0.35 mm out. dia., EICOM), was inserted into the guide cannula and locked in position with a cap nut (EICOM). Thereafter, the rats were kept in individual cages.

35           About a week of feeding after implantation of the guide cannula for postoperative recuperation, an operation was performed for measurements of blood



pressure in conscious state. The rat described above was anesthetized with pentobarbital 50 mg/kg i.p. and immobilized in spine position on a necropsy pad and the left femoral artery was exposed. Polyethylene tubing, SP35 (0.5 mm in. dia., 0.9 mm out. dia., Natsume Seisakusho), was cut to about 60 cm in length and filled with 200 U/ml heparin-containing saline. This tube was inserted about 2.5 cm deep into the femoral artery and secured in position. The free end of the tube was passed under the dorsal skin and exposed in the cervical region (dorsal side).

After waiting overnight postoperatively, the polyethylene tube was connected to a transducer (Spectramed) and the blood pressure was measured. After blood pressure readings became steady, the cap nut and dummy cannula were removed from the rat skull and, instead, a stainless steel microinjection cannula (0.17 mm in. dia., 0.35 mm out. dia., EICOM) connected to a Teflon tube (50 cm long, 0.1 mm in. dia., 0.4 mm out. dia., EICOM) was inserted. The length of the microinjection cannula was adjusted beforehand so that its tip would extend 1 mm from the guide cannula (Fig. 46). One end of the Teflon tube was connected to a microsyringe pump and either PBS or 19P2-L31 dissolved in PBS was injected, in a total volume of 2  $\mu$ l, into the area postrema at a flow rate of 1.0  $\mu$ l/min.

After measurement of blood pressure, the microinjection cannula used for injection of 19P2-L31 was disconnected and replaced with a microinjection cannula for injection of a stain (Evans Blue) solution. The stain was infused at the same rate of 1.0  $\mu$ l/min as the injection of 19P2-L31 for 2 minutes. After a standby time of about 3 minutes, the microinjection cannula was disconnected. The rat was decapitated and the brain was quickly removed and frozen. The brains were cut serial frontal sections on cryostat and the position of

dye infusion was confirmed.

Results of the above experiment showed that injection of 10 nmol of 19P2-L31 into the area postrema of medula oblongata caused an elevation of blood pressure. Typical examples of direct and mean blood pressure are shown in Fig. 47.

[Example 41]

Effects of ligand polypeptide on plasma pituitary hormone level

10 The inventors explored the effect of 19P2-L31 administered into the third ventricle on pituitary hormone levels in the plasma. Mature male Wistar rats (body weights at operation: 290-350 g) were anesthetized with pentobarbital, 50 mg/kg i.p., and each immobilized in a rat brain stereotaxic apparatus. The incisor bar was set 3.3 mm lower from the interaural line. The skull was exposed, and using a dental drill a hole was made on the bone for implantation of a guide cannula. In addition, an anchor screw was buried in one position around the hole. A stainless-steel guide cannula, AG-12 (0.4 mm in. dia., 0.5 mm out. dia., EICOM), was inserted in such a manner that its tip would be situated in the upper part of the third ventricle. With reference to 20 the atlas of Paxinos and Watson (1986), the stereotaxic coordinates were AP: +7.2 mm (from interaural line), L: 0.0 mm, and H: +2.0 mm (from interaural line). The guide cannula was secured to the skull using an instant adhesive, a dental cement, and an anchor piece. A stainless-steel dummy cannula, AD-12 (0.35 mm out. 30 dia., EICOM), was then passed through the guide cannula and locked in position with a cap nut (EICOM). After the operation the rats were housed in individual cages and kept for at least 3 days for recuperation before starting the experiment.

The operated rat was anesthetized with

pentobarbital 50 mg/kg i.p. and immobilized in dorsal position. After the bilateral jugular veins were exposed, 400  $\mu$ l of blood was drawn using a 1 ml tuberculin syringe and a 24-G needle (both by Termo).

5 To prevent clotting, the syringe was filled with 20  $\mu$ l of saline containing 200 U/ml of heparin beforehand. The cap nut and dummy cannula were removed from the rat skull and, instead, a stainless steel microinjection cannula (0.17 mm in. dia., 0.35 mm out. dia., EICOM)

10 connected to Teflon tube (50 cm long, 0.1 mm in. dia., 0.4 mm out. dia., EICOM) was inserted. The length of the microinjection cannula was adjusted beforehand so that its tip would be emergent from the guide cannula by 1 mm. One end of the Teflon tube was connected to a

15 microsyringe pump and either PBS or 19P2-L31 dissolved in PBS was injected, in a total volume of 10  $\mu$ l, into the third ventricle at a flow rate of 2.5  $\mu$ l/min. After a standby time of 1 minute following infusion, the microinjection cannula was disconnected and the

20 dummy cannula was reinstated and locked in position with a cap nut. Immediately before initiation of intraventricular administration and 10, 20, 30, 40, and 60 minutes after initiation of administration, 400  $\mu$ l portions of blood were drawn from the jugular vein.

25 Each blood sample was centrifuged (5,000 rpm, 10 min.) with a high-speed refrigerated microcentrifuge (MR-150, Tommy Seiko) and the supernatant (plasma) was recovered. The amounts of pituitary hormones [prolactin, luteinizing hormone (LH),

30 adrenocorticotrophic hormone (ACTH), thyroid-stimulating hormone (TSH), and growth hormone (GH)] in the plasma were respectively determined by radioimmunoassays.

The results were expressed as a mean  $\pm$  S.E.M. To test for significant difference between the group

35 treated with 19P2-L31 dissolved in PBS and the control group treated with PBS alone, Student's t-test was

used. According to the two-tailed test,  $p < 0.05$  was assumed to be the minimal level of significance. As shown in Fig. 48, the plasma GH level was significantly decreased at 20 minutes after administration of 50 nmol of 19P2-L31 into the third ventricle, as compared with the control group. Tendencies toward decrease were found at 10, 30, and 40 minutes after administration as well but the changes were not statistically significant. At 60 minutes after administration, there was no difference from the control group. As to plasma prolactin, LH, ACTH, and TSH, none showed significant changes.

[Example 42]

Effects of ligand polypeptide on plasma growth hormone (GH) level in freely moving rats

Mature male Wistar rats were anesthetized with pentobarbital 50 mg/kg i.p. and, as in Example 41, a stainless-steel guide cannula AG-12 (0.4 mm in. dia., 0.5 mm out. dia., EICOM) was implanted in position with its tip situated in the upper part of the third ventricle. After the operation the rats were housed in individual cages and kept for at least 3 days for recuperation and, then, a cannula (30 cm long, 0.5 mm in. dia., 0.9 mm out. dia., Natsume Seisakusho) filled with heparin (200 U/ml)-containing saline was inserted into the right atrium from the right jugular vein under pentobarbital anesthesia. The rats were maintained overnight for complete arousal from anesthesia and then transferred to transparent acrylic cages (30 cm x 30 cm x 35 cm). A 1 ml tuberculin syringe with a 24-G needle (both by Terumo) was connected to the cannula inserted in the atrium and 300  $\mu$ l of blood was drawn. To prevent clotting, the syringe was filled with 20  $\mu$ l of saline containing 200 U/ml of heparin beforehand. A stainless-steel microinjection cannula (0.17 mm in. dia., 0.35 mm out. dia., EICOM) connected to Teflon

tube (50 cm long, 0.1 mm in. dia., 0.4 mm out. dia.,  
 EICOM) was inserted into the guide cannula positioned  
 in the third ventricle. The length of the  
 microinjection cannula was adjusted beforehand so that  
 5 its tip would be extend 1 mm from the guide cannula.  
 One end of the Teflon tube was connected to a  
 microsyringe pump and either PBS or 19P2-L31 dissolved  
 in PBS was injected, in a total volume of 10  $\mu$ l, into  
 the third ventricle at a flow rate of 2.5  $\mu$ l/min. Ten  
 10 minutes after initiation of administration into the  
 third ventricle, 5  $\mu$ g/kg GHRH-saline was administered  
 via the cannula inserted into the atrium. Immediately  
 before initiation of intraventricular administration  
 and 10, 20, 30, 40, and 60 minutes after administration  
 15 of GHRH, 300  $\mu$ l portions of blood were drawn from the  
 jugular vein. Each blood sample was centrifuged (5,000  
 rpm, 10 min.) and the supernatant (plasma) was  
 recovered. The concentrations of GH in the plasma were  
 determined by radioimmunoassay.

20 The results were expressed as a mean  $\pm$  S.E.M. To  
 test for significant difference between the group  
 treated with 19P2-L31 dissolved in PBS and the control  
 group treated with PBS alone, Student's t-test was  
 used. According to the two tailed test,  $p < 0.05$  was  
 25 assumed to be the minimal level of significance. As  
 shown in Fig. 49, administration of 5  $\mu$ g/kg of GHRH  
 elevated the plasma GH level. However, when 50 nmol of  
 19P2-L31 was administered into the third ventricle, the  
 GHRH-induced elevation of plasma GH was significantly  
 30 inhibited.

[Example 43]

Preparation of rabbit anti-bovine 19P2-L31  
 antibodies

35 Synthetic peptides containing partial 19P2-L31  
 sequence [peptide-I: SRAHQHSMEIRTPDC (SEQ ID NO:92),  
 peptide-II: CAWYAGRGIRPVGRFNH<sub>2</sub> (SEQ ID NO:93), and

peptide-III: CEIRTPDINPAWYAG (SEQ ID NO:94) were conjugated with KLH according to the standard method. Each peptide conjugate (600 µg as a peptide) dissolved in saline was mixed with Freund's complete adjuvant, and the resultant emulsion was subcutaneously injected into three rabbits (NZW, male, 2.5 kg) respectively. Hyperimmunization was carried out three times in total at the same dose of the conjugate as the first injection with Freund's incomplete adjuvant every three weeks. Antibody titers were determined as follows. Two weeks after the last immunization, blood samples were obtained from the vein of the immunized rabbits respectively. After being incubated at 37°C for 1 hour, the blood samples were kept at 4°C over night. Sera were then prepared by means of centrifugation. An aliquot (100 µl) of each serum sample diluted properly was introduced into 96-well polystyrene microplates which were pre-coated with goat anti-rabbit IgG (Fc) antibodies, and then the microplates were incubated at 4°C for 16 hours. After removing the sera, horse radish peroxidase (HRP)-conjugated peptide-I, II, and III were added to the wells respectively, and then the microplates were incubated at room temperature for 4 hours. After removing the peptides, coloring reaction was done by adding a substrate. The reaction was stopped by adding 100 µl of a stopping solution, and then the absorbance at 450 nm in each well was measured. As shown in Fig. 50, serum samples obtained from the rabbits after the immunization showed binding activities to HRP-conjugated peptides respectively. However, none of binding activities was detected in sera prepared before the immunization. These results indicated that the rabbits received the immunization produced antibodies against peptide-I, II, and III, respectively. To prepare purified IgG antibody fractions, sera obtained from the immunized rabbits was

precipitated with ammonium sulfate. The resultant precipitates were dissolved in borate buffer, and then dialyzed with the same buffer. The IgG fractions thus obtained were then subjected onto affinity columns conjugated with peptide-I or 19P2-L31 respectively. After washing the columns with borate buffer and following with acetate buffer (100 mM, pH 4.5), antibodies bound to the column were eluted with glycine buffer (200 mM, pH 2.0). After being neutralized with 1M Tris, the eluents were used as purified antibodies respectively.

[Example 44]

Inhibitory activity of antibodies against the release of arachidonic acid metabolites induced by 19P2-L31

The purified antibodies prepared as described in Example 43 were tested their inhibitory activity against the release of arachidonic acid metabolites induced by 19P2-L31. The antibodies diluted as indicated in Fig. 51 were mixed with 19P2-L31 ( $5 \times 10^{-10}$  M) at room temperature for 1 hour, and then the release of arachidonic acid metabolites was examined as described in Example 11. As shown in Fig. 51, the highest inhibitory activity was observed in anti-peptide-II antibodies.

[Preparation Example 1]

Fifty milligrams of the compound as obtained in Example 21 is dissolved in 50 ml of Japanese pharmacopoeial, distilled water for injection, and Japanese pharmacopoeial, distilled water for injection is added thereto to make 100 ml. The resulting solution is filtered under a germ-free condition, and the filtrate of 1 ml each is filled in vials for injection, freeze-dried and sealed therein also under a germ-free condition.

[Preparation Example 2]

One hundred milligrams of the compound as obtained in Example 21 is dissolved in 50 ml of Japanese pharmacopoeial, distilled water for injection, and Japanese pharmacopoeial, distilled water for injection is added thereto to make 100 ml. The resulting solution is filtered under a germ-free condition, and the filtrate of 1 ml each is filled in vials for injection, freeze-dried and sealed therein also under a germ-free condition.

[Evaluation of the physiological activities of ligand polypeptide of the present invention]

The above examples 37-41 demonstrate that topical administration of ligand polypeptide induces enhancement of spontaneous motor activity and rearing behavior, elevation of body temperature and blood pressure, and decrease in plasma growth hormone concentration. These findings relating to physiological activities are the first proof of various prominent physiologic changes which occur when ligand polypeptide acts on the central nervous system.

Since ligand polypeptide of the present invention, inclusive of its salt, acts on the central nervous systems of warm-blooded animals (e.g. rat, mouse, guinea pig, chicken, rabbit, dog, swine, bovine, sheep, monkey, and man) to induce a variety of pharmacological changes, it is showed that the ligand and salt have the property to alter the intracranial nervous system and endocrine system.

When 19P2-L31 was administered into the lateral ventricle of mice, an increase in the amount of activity was found at the level of 0.01-10 nmol. This fact shows that ligand polypeptide triggers changes in the motor system via the G protein-coupled receptors of the central nervous system. It was also found that administration of the peptide into the lateral



ventricle of mice results in elevation of body temperature and that administration into the area postrema of medula oblongata of rats results in elevation of blood pressure. These actions resemble the pharmacologic actions of known central stimulants (e.g. amphetamine, cocaine, methylphenidate, etc.). Therefore, it is showed that ligand polypeptide or a salt thereof releases biologic amines (dopamine, noradrenaline, serotonin) from the nerve ending reservoirs, in the main (Michio Yuzuru and Takeo Yoshikawa, Medical Science, 42, 535-536, 1991).

Furthermore, when 19P2-L31 was injected into the third ventricle of rats, the plasma growth hormone level was depressed. This finding shows that this peptide acts on the hypothalamus and is associated with secretion of pituitary hormones via the hypothalamo-pituitary system. It is also possible that this peptide directly act on the pituitary so as to suppress the release of growth hormone. Growth hormone releasing hormone (GHRH) which regulates secretion of growth hormone from the hypophysis as well as somatostatin exists in the neighborhood of the third ventricle (Masahiro Tohyama et al., Kagakuteki Shinkeikino Kaibogaku (Chemical Neuroanatomy), 167-216, 1987). Therefore, it is showed that 19P2-L31 is modulating release of these substances.

The above facts show that ligand polypeptide is a peptide acting on the central nervous system to control the autonomous nervous system. The fact that the mRNA of this peptide and of its receptor is expressed at high levels in the hypothalamus and medula oblongata also shows the involvement of ligand polypeptide in the modulation of the autonomous nervous system. In fact, the superior center of autonomous nerve peripherals is the medula oblongata and hypothalamus, where as already elucidated the sympathetic nervous system and the para-

sympathetic nervous system are integrated to play an important role in both neural regulation and humoral regulation.

The above findings indicate the usefulness of  
 5 ligand polypeptide or an agonist of ligand polypeptide,  
 or a salt thereof, as a central nervous system  
 stimulant causing enhancement of spontaneous motor  
 activity. Thus, the peptide can be used as a  
 prophylactic and/or therapeutic drug for a variety of  
 10 diseases such as senile dementia, cerebrovascular  
 dementia (dementia due to cerebrovascular disorder),  
 dementia associated with phylodegenerative retroplastic  
 diseases (e.g. Alzheimer's disease, Parkinson's  
 disease, Pick's disease, Huntington's disease, etc.),  
 15 dementia due to infectious diseases (e.g. delayed viral  
 infections such as Creutzfeldt-Jakob disease), dementia  
 associated with endocrine, metabolic, and toxic  
 diseases (e.g. hypothyroidism, vitamin B12 deficiency,  
 alcoholism, and poisoning due to various drugs, metals,  
 20 or organic compounds), dementia associated with  
 oncogenous diseases (e.g. brain tumor), dementia due to  
 traumatic diseases (e.g. chronic subdural hematoma),  
 depression (melancholia), hyperkinetic (microencephalo-  
 pathy) syndrome, or disturbance of consciousness. On  
 25 the other hand, an antagonist of 19P2 ligand or a salt  
 thereof is of value as a CNS depressant, for instance,  
 and can be used as an antipsychotic drug, an anti-  
 Huntigton's disease drug, an antianxiety drug, or a  
 hypnotic-sedative.

30 It was made clear that injection of ligand  
 polypeptide into the area postrema of medula oblongata  
 elevates the blood pressure. Therefore, ligand  
 polypeptide or an agonist of ligand polypeptide, or a  
 salt thereof, is of value as a vasopressor. On the  
 35 other hand, a ligand polypeptide antagonist or a salt  
 thereof is of value as a depressor.

It was found that when ligand polypeptide acts on the hypothalamus, the plasma growth hormone level is depressed. Hypersecretion of growth hormone triggers somatomegaly and acromegalic gigantism (Katamasu et al., Endocrine Syndrome, 78-80, 1993; Hiroi et al., Endocrine Syndrome, 149-151, 1993). Therefore, ligand polypeptide or a ligand polypeptide antagonist, or a salt thereof, can be used as a prophylactic and/or therapeutic drug for somatomegaly and acromegalic gigantism. Moreover, growth hormone promotes release of glucose from the liver and inhibits the uptake of glucose by muscles and adipose tissues from the blood, causing hyperglycemia and diabetes [Eiji Kobayashi, Naibumpi Gensho (Endocrine Phenomena), 1980]. In fact, the secretion of growth hormone is elevated in diabetic patients (Hiroshi Kiyono, Endocrinology and Metabolic Diseases, 385-402, 1994). Therefore, ligand polypeptide or an agonist of ligand polypeptide, or a salt thereof, can be used as a prophylactic and/or therapeutic drug for diabetes, for instance.

On the other hand, an antagonist of ligand polypeptide promotes secretion of growth hormone. Therefore, a ligand polypeptide antagonist or a salt thereof can be used as a prophylactic and/or therapeutic drug for pituitarism leading to a depressed growth hormone level, pituitary dwarfism, and hypoglycemia. Moreover, growth hormone and insulin-like growth factor secreted by growth hormone are effective in amyotrophic lateral sclerosis, osteoporosis, renal failure, and improvement in postoperative nutritional status (Shizume et al., Endocrine Syndrome, 84-87, 1993, Nikkei Bio-Annal 96, 453-454, 1996; Tobiume et al., Clinical Endocrinology, 44, 1205-1214, 1996). Therefore, a ligand polypeptide antagonist or its salt can be used as a prophylactic and/or therapeutic drug for such illnesses.

## [Sequence Listing]

## (1) GENERAL INFORMATION:

## (i) APPLICANT:

5 (A) NAME: Takeda Chemical Industries, Ltd.  
 (B) STREET: 1-1, Doshomachi 4-chome, Chuo-ku  
 (C) CITY: Osaka  
 (D) STATE: Osaka  
 (E) COUNTRY: Japan  
 (F) POSTAL CODE (ZIP): 541

10 (ii) TITLE OF INVENTION: Polypeptides, Their Production  
 and Use

(iii) NUMBER OF SEQUENCES: 94

## (iv) COMPUTER READABLE FORM:

15 (A) MEDIUM TYPE:  
 (B) COMPUTER:  
 (C) OPERATING SYSTEM:  
 (D) SOFTWARE:

## (v) CURRENT APPLICATION DATA:

APPLICATION NUMBER:

20

## (2) INFORMATION FOR SEQ ID NO:1:

## (i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 98  
 (B) TYPE: Amino acid  
 25 (C) TOPOLOGY: Linear

(ii) MOLECULE TYPE: Peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

Met Lys Ala Val Gly Ala Trp Leu Leu Cys Leu Leu Leu Gly Leu  
 30 1 5 10 15  
 Ala Leu Gln Gly Ala Ala Ser Arg Ala His Gln His Ser Met Glu Ile  
 20 25 30  
 Arg Thr Pro Asp Ile Asn Pro Ala Trp Tyr Ala Gly Arg Gly Ile Arg  
 35 40 45  
 35 Pro Val Gly Arg Phe Gly Arg Arg Ala Ala Pro Gly Asp Gly Pro  
 50 55 60

Arg Pro Gly Pro Arg Arg Val Pro Ala Cys Phe Arg Leu Glu Gly Gly  
 65 70 75 80  
 Ala Glu Pro Ser Arg Ala Leu Pro Gly Arg Leu Thr Ala Gln Leu Val  
 85 90 95  
 5 Gln Glu

## (2) INFORMATION FOR SEQ ID NO:2:

## (i) SEQUENCE CHARACTERISTICS:

10 (A) LENGTH: 294  
 (B) TYPE: Nucleic acid  
 (C) STRANDEDNESS: Double  
 (D) TOPOLOGY: Linear

(ii) MOLECULE TYPE: cDNA

(xi) FEATURE

15 (C) IDENTIFICATION METHOD: S

(x) SEQUENCE DESCRIPTION; SEQ ID NO:2:

ATGAAGGCGG TGGGGGCGCTG GCTCCTCTGC CTGCTGCTGC TGGGCCTGGC CCTGCAGGGG 60  
 GCTGCCAGCA GAGCCACCA GCACTCCATG GAGATCCGCA CCCCCGACAT CAACCCTGCC 120  
 20 TGGTACGCRG GCCGTGGGAT CCGGCCCGTG GGCCGCTTCG GCCGGCGAAG AGCTGCCCCY 180  
 GGGGACGGAC CCAGGCCTGG CCCCCGGCGT GTGCCGGCCT GCTTCCGCCT GGAAGCGGGY 240  
 GCTGAGCCCT CCCGAGCCCT CCCGGGGCGG CTGACGGCCC AGCTGGTCCA GGAA 294

## (2) INFORMATION FOR SEQ ID NO:3:

25 (i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 29  
 (B) TYPE: Amino acid  
 (C) TOPOLOGY: Linear

(ii) MOLECULE TYPE: Peptide

30 (xi) SEQUENCE DESCRIPTION: SEQ ID NO:3:

Ser Arg Ala His Gln His Ser Met Glu Ile Arg Thr Pro Asp Ile Asn  
 1 5 10 15  
 Pro Ala Trp Tyr Ala Gly Arg Gly Ile Arg Pro Val Gly  
 35 20 25

## (2) INFORMATION FOR SEQ ID NO:4:

## (i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 19

(B) TYPE: Amino acid

5 (C) TOPOLOGY: Linear

(ii) MOLECULE TYPE: Peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:4:

Thr Pro Asp Ile Asn Pro Ala Trp Tyr Ala Gly Arg Gly Ile Arg Pro  
 10 1 5 10 15  
 Val Gly Arg  
 19

## (2) INFORMATION FOR SEQ ID NO:5:

## 15 (i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 31

(B) TYPE: Amino acid

(C) TOPOLOGY: Linear

(ii) MOLECULE TYPE: Peptide

20 (xi) SEQUENCE DESCRIPTION: SEQ ID NO:5:

Ser Arg Ala His Gln His Ser Met Glu Ile Arg Thr Pro Asp Ile Asn  
 1 5 10 15  
 Pro Ala Trp Tyr Ala Gly Arg Gly Ile Arg Pro Val Gly Arg Phe  
 25 20 25 30

## (2) INFORMATION FOR SEQ ID NO:6:

## (i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 32

30 (B) TYPE: Amino acid

(C) TOPOLOGY: Linear

(ii) MOLECULE TYPE: Peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:6:

35 Ser Arg Ala His Gln His Ser Met Glu Ile Arg Thr Pro Asp Ile Asn  
 1 5 10 15

Pro Ala Trp Tyr Ala Gly Arg Gly Ile Arg Pro Val Gly Arg Phe Gly  
                   20                  25                  30

## (2) INFORMATION FOR SEQ ID NO:7:

- 5 (i) SEQUENCE CHARACTERISTICS:  
       (A) LENGTH: 33  
       (B) TYPE: Amino acid  
       (C) TOPOLOGY: Linear  
      (ii) MOLECULE TYPE: Peptide  
 10 (xi) SEQUENCE DESCRIPTION: SEQ ID NO:7:

Ser Arg Ala His Gln His Ser Met Glu Ile Arg Thr Pro Asp Ile Asn  
      1                  5                  10                  15  
 Pro Ala Trp Tyr Ala Gly Arg Gly Ile Arg Pro Val Gly Arg Phe Gly  
 15                  20                  25                  30  
 Arg  
 33

## (2) INFORMATION FOR SEQ ID NO:8:

- 20 (i) SEQUENCE CHARACTERISTICS:  
       (A) LENGTH: 20  
       (B) TYPE: Amino acid  
       (C) TOPOLOGY: Linear  
      (ii) MOLECULE TYPE: Peptide  
 25 (xi) SEQUENCE DESCRIPTION: SEQ ID NO:8:

Thr Pro Asp Ile Asn Pro Ala Trp Tyr Ala Gly Arg Gly Ile Arg Pro  
      1                  5                  10                  15  
 Val Gly Arg Phe  
 30                  20

## (2) INFORMATION FOR SEQ ID NO:9:

- (i) SEQUENCE CHARACTERISTICS:  
       (A) LENGTH: 21  
 35 (B) TYPE: Amino acid  
       (C) TOPOLOGY: Linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:9:

(2) INFORMATION FOR SEQ ID NO:10:

(C) TOPOLOGY: Linear

15 (xi) SEQUENCE DESCRIPTION: SEQ ID NO:10:

(2) INFORMATION FOR SEQ ID NO:11:

(D) TOPOLOGY: Linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:11:

(2) INFORMATION FOR SEQ ID NO:12:

(A) LENGTH: 57



(B) TYPE: Nucleic acid  
 (C) STRANDEDNESS: Double  
 (D) TOPOLOGY: Linear  
 (ii) MOLECULE TYPE: cDNA  
 5 (xi) SEQUENCE DESCRIPTION: SEQ ID NO:12:

ACCCCCGACA TCAACCCTGC CTGGTACGCR GGCCGTGGGA TCGGCCCCGT GGGCCGC 57

(2) INFORMATION FOR SEQ ID NO:13:

10 (i) SEQUENCE CHARACTERISTICS:  
 (A) LENGTH: 93  
 (B) TYPE: Nucleic acid  
 (C) STRANDEDNESS: Double  
 (D) TOPOLOGY: Linear  
 15 (ii) MOLECULE TYPE: cDNA  
 (xi) SEQUENCE DESCRIPTION: SEQ ID NO:13:

AGCAGAGCCC ACCAGCACTC CATGGAGATC CGCACCCCCG ACATCAACCC TGCCTGGTAC 60  
 GCRGGCCGTG GGATCCGGCC CGTGGGCCGC TTC 93

20

(2) INFORMATION FOR SEQ ID NO:14:

(i) SEQUENCE CHARACTERISTICS:  
 (A) LENGTH: 96  
 (B) TYPE: Nucleic acid  
 25 (C) STRANDEDNESS: Double  
 (D) TOPOLOGY: Linear  
 (ii) MOLECULE TYPE: cDNA  
 (xi) SEQUENCE DESCRIPTION: SEQ ID NO:14:

30 AGCAGAGCCC ACCAGCACTC CATGGAGATC CGCACCCCCG ACATCAACCC TGCCTGGTAC 60  
 GCRGGCCGTG GGATCCGGCC CGTGGGCCGC TTCGCC 96

(2) INFORMATION FOR SEQ ID NO:15:

(i) SEQUENCE CHARACTERISTICS:  
 35 (A) LENGTH: 99  
 (B) TYPE: Nucleic acid

(C) STRANDEDNESS: Double  
 (D) TOPOLOGY: Linear  
 (ii) MOLECULE TYPE: cDNA  
 (xi) SEQUENCE DESCRIPTION: SEQ ID NO:15:

5

AGCAGAGCCC ACCAGCACTC CATGGAGATC CGCACCCCGG ACATCAACCC TGCCTGGTAC 60  
 GCRGGCCGTG GGATCCGGCC CGTGGGCCGC TTCGGCCGG 99

## (2) INFORMATION FOR SEQ ID NO:16:

10

## (i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 60  
 (B) TYPE: Nucleic acid  
 (C) STRANDEDNESS: Double  
 (D) TOPOLOGY: Linear

15

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:16:

ACCCCGGACA TCAACCCTGC CTGGTACGCR GGCCGTGGGA TCCGGCCCGT GGGCCGCTTC 60

20

## (2) INFORMATION FOR SEQ ID NO:17:

## (i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 63  
 (B) TYPE: Nucleic acid  
 (C) STRANDEDNESS: Double  
 (D) TOPOLOGY: Linear

25

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:17:

ACCCCGGACA TCAACCCTGC CTGGTACGCR GGCCGTGGGA TCCGGCCCGT GGGCCGCTTC 60  
 GGC 63

30

## (2) INFORMATION FOR SEQ ID NO:18:

## (i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 66  
 (B) TYPE: Nucleic acid  
 (C) STRANDEDNESS: Double

35

(D) TOPOLOGY: Linear  
(ii) MOLECULE TYPE: cDNA  
(xi) SEQUENCE DESCRIPTION: SEQ ID NO:18:

5 ACCCCCGACA TCAACCCTGC CTGGTACGCR GGCCGTGGGA TCCGGCCCGT GGGCCGCTTC 60  
GGCCGG 66

(2) INFORMATION FOR SEQ ID NO:19:

(i) SEQUENCE CHARACTERISTICS:

10 (A) LENGTH: 91  
(B) TYPE: Amino acid  
(C) TOPOLOGY: Linear  
(ii) MOLECULE TYPE: Peptide  
(xi) SEQUENCE DESCRIPTION: SEQ ID NO:19:

15 Leu Val Leu Val Ile Ala Arg Val Arg Arg Leu His Asn Val Thr Asn  
1 5 10 15  
Phe Leu Ile Gly Asn Leu Ala Leu Ser Asp Val Leu Met Cys Thr Ala  
20 25 30  
20 Cys Val Pro Leu Thr Leu Ala Tyr Ala Phe Glu Pro Arg Gly Trp Val  
35 40 45  
Phe Gly Gly Gly Leu Cys His Leu Val Phe Phe Leu Gln Pro Val Thr  
50 55 60  
Val Tyr Val Ser Val Phe Thr Leu Thr Thr Ile Ala Val Asp Arg Tyr  
25 65 70 75 80  
Val Val Leu Val His Pro Leu Arg Arg Arg Ile  
85 90

(2) INFORMATION FOR SEQ ID NO:20:

(i) SEQUENCE CHARACTERISTICS:

30 (A) LENGTH: 59  
(B) TYPE: Amino acid  
(C) TOPOLOGY: Linear  
(ii) MOLECULE TYPE: Peptide  
35 (xi) SEQUENCE DESCRIPTION: SEQ ID NO:20:

Gly Leu Leu Leu Val Thr Tyr Leu Leu Pro Leu Leu Val Ile Leu Leu  
 1 5 10 15  
 Ser Tyr Val Arg Val Ser Val Lys Leu Arg Asn Arg Val Val Pro Gly  
 20 25 30  
 5 Cys Val Thr Gln Ser Gln Ala Asp Trp Asp Arg Ala Arg Arg Arg Arg  
 35 40 45  
 Thr Phe Cys Leu Leu Val Val Val Val Val Val  
 50 55

10 (2) INFORMATION FOR SEQ ID NO:21:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 370

(B) TYPE: Amino acid

(C) TOPOLOGY: Linear

15 (ii) MOLECULE TYPE: Peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:21:

Met Ala Ser Ser Thr Thr Arg Gly Pro Arg Val Ser Asp Leu Phe Ser  
 1 5 10 15  
 20 Gly Leu Pro Pro Ala Val Thr Thr Pro Ala Asn Gln Ser Ala Glu Ala  
 20 25 30  
 Ser Ala Gly Asn Gly Ser Val Ala Gly Ala Asp Ala Pro Ala Val Thr  
 35 40 45  
 Pro Phe Gln Ser Leu Gln Leu Val His Gln Leu Lys Gly Leu Ile Val  
 25 50 55 60  
 Leu Leu Tyr Ser Val Val Val Val Val Gly Leu Val Gly Asn Cys Leu  
 65 70 75 80  
 Leu Val Leu Val Ile Ala Arg Val Arg Arg Leu His Asn Val Thr Asn  
 85 90 95  
 30 Phe Leu Ile Gly Asn Leu Ala Leu Ser Asp Val Leu Met Cys Thr Ala  
 100 105 110  
 Cys Val Pro Leu Thr Leu Ala Tyr Ala Phe Glu Pro Arg Gly Trp Val  
 115 120 125  
 Phe Gly Gly Gly Leu Cys His Leu Val Phe Phe Leu Gln Pro Val Thr  
 35 130 135 140  
 Val Tyr Val Ser Val Phe Thr Leu Thr Thr Ile Ala Val Asp Arg Tyr

145                      150                      155                      160  
 Val Val Leu Val His Pro Leu Arg Arg Arg Ile Ser Leu Arg Leu Ser  
                          165                      170                      175  
 Ala Tyr Ala Val Leu Ala Ile Trp Ala Leu Ser Ala Val Leu Ala Leu  
 5                      180                      185                      190  
 Pro Ala Ala Val His Thr Tyr His Val Glu Leu Lys Pro His Asp Val  
                          195                      200                      205  
 Arg Leu Cys Glu Glu Phe Trp Gly Ser Gln Glu Arg Gln Arg Gln Leu  
                          210                      215                      220  
 10 Tyr Ala Trp Gly Leu Leu Leu Val Thr Tyr Leu Leu Pro Leu Leu Val  
                          225                      230                      235                      240  
 Ile Leu Leu Ser Tyr Val Arg Val Ser Val Lys Leu Arg Asn Arg Val  
                          245                      250                      255  
 Val Pro Gly Cys Val Thr Gln Ser Gln Ala Asp Trp Asp Arg Ala Arg  
 15                      260                      265                      270  
 Arg Arg Arg Thr Phe Cys Leu Leu Val Val Val Val Val Val Phe Ala  
                          275                      280                      285  
 Val Cys Trp Leu Pro Leu His Val Phe Asn Leu Leu Arg Asp Leu Asp  
                          290                      295                      300  
 20 Pro His Ala Ile Asp Pro Tyr Ala Phe Gly Leu Val Gln Leu Leu Cys  
                          305                      310                      315                      320  
 His Trp Leu Ala Met Ser Ser Ala Cys Tyr Asn Pro Phe Ile Tyr Ala  
                          325                      330                      335  
 Trp Leu His Asp Ser Phe Arg Glu Glu Leu Arg Lys Leu Leu Val Ala  
 25                      340                      345                      350  
 Trp Pro Arg Lys Ile Ala Pro His Gly Gln Asn Met Thr Val Ser Val  
                          355                      360                      365  
 Val Ile  
                          370

30

## (2) INFORMATION FOR SEQ ID NO:22:

## (i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 206

(B) TYPE: Amino acid

35 (C) TOPOLOGY: Linear

(ii) MOLECULE TYPE: Peptide

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:22:

Leu Val Leu Val Ile Ala Arg Val Arg Arg Leu Tyr Asn Val Thr Asn  
 1 5 10 15  
 5 Phe Leu Ile Gly Asn Leu Ala Leu Ser Asp Val Leu Met Cys Thr Ala  
 20 25 30  
 Cys Val Pro Leu Thr Leu Ala Tyr Ala Phe Glu Pro Arg Gly Trp Val  
 35 40 45  
 Phe Gly Gly Gly Leu Cys His Leu Val Phe Phe Leu Gln Ala Val Thr  
 10 50 55 60  
 Val Tyr Val Ser Val Phe Thr Leu Thr Thr Ile Ala Val Asp Arg Tyr  
 65 70 75 80  
 Val Val Leu Val His Pro Leu Arg Arg Arg Ile Ser Leu Arg Leu Ser  
 85 90 95  
 15 Ala Tyr Ala Val Leu Ala Ile Trp Val Leu Ser Ala Val Leu Ala Leu  
 100 105 110  
 Pro Ala Ala Val His Thr Tyr His Val Glu Leu Lys Pro His Asp Val  
 115 120 125  
 Arg Leu Cys Glu Glu Phe Trp Gly Ser Gln Glu Arg Gln Arg Gln Leu  
 20 130 135 140  
 Tyr Ala Trp Gly Leu Leu Leu Val Thr Tyr Leu Leu Pro Leu Leu Val  
 145 150 155 160  
 Ile Leu Leu Ser Tyr Ala Arg Val Ser Val Lys Leu Arg Asn Arg Val  
 165 170 175  
 25 Val Pro Gly Arg Val Thr Gln Ser Gln Ala Asp Trp Asp Arg Ala Arg  
 180 185 190  
 Arg Arg Arg Thr Phe Cys Leu Leu Val Val Val Val Val Val  
 195 200 205

## 30 (2) INFORMATION FOR SEQ ID NO:23:

## (i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 126  
 (B) TYPE: Amino acid  
 (C) TOPOLOGY: Linear

## 35 (ii) MOLECULE TYPE: Peptide

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:23:

Val Val Leu Val His Pro Leu Arg Arg Arg Ile Ser Leu Arg Leu Ser  
 1 5 10 15  
 Ala Tyr Ala Val Leu Gly Ile Trp Ala Leu Ser Ala Val Leu Ala Leu  
 20 25 30  
 5 Pro Ala Ala Val His Thr Tyr His Val Glu Leu Lys Pro His Asp Val  
 35 40 45  
 Ser Leu Cys Glu Glu Phe Trp Gly Ser Gln Glu Arg Gln Arg Gln Ile  
 50 55 60  
 Tyr Ala Trp Gly Leu Leu Leu Gly Thr Tyr Leu Leu Pro Leu Leu Ala  
 10 65 70 75 80  
 Ile Leu Leu Ser Tyr Val Arg Val Ser Val Lys Leu Arg Asn Arg Val  
 85 90 95  
 Val Pro Gly Ser Val Thr Gln Ser Gln Ala Asp Trp Asp Arg Ala Arg  
 100 105 110  
 15 Arg Arg Arg Thr Phe Cys Leu Leu Val Val Val Val Val Val  
 115 120 125

## (2) INFORMATION FOR SEQ ID NO:24:

## (i) SEQUENCE CHARACTERISTICS:

20 (A) LENGTH: 273  
 (B) TYPE: Nucleic acid  
 (C) STRANDEDNESS: Double  
 (D) TOPOLOGY: Linear

(ii) MOLECULE TYPE: cDNA

25 (ix) FEATURE

(C) IDENTIFICATION METHOD: S

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:24:

CTGGTGTCTGG TGATCGCGCG GGTGCGCCGG CTGCACAACG TGACGAACTT CCTCATCGGC 60  
 30 AACCTGGCCT TGTCCGACGT GCTCATGTGC ACCGCCTGCG TGCCGCTCAC GCTGGCCTAT 120  
 GCCTTCGAGC CACGCGGCTG GGTGTTCCGGC GGCGGCCTGT GCCACCTGGT CTTCTTCCTG 180  
 CAGCCGGTCA CCGTCTATGT GTCGGTGTTC ACGCTCACCA CCATCGCAGT GGACCGGTAC 240  
 GTCGTGCTGG TGCACCCGCT GAGGCGGCGC ATC 273

## 35 (2) INFORMATION FOR SEQ ID NO:25:

## (i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 177  
 (B) TYPE: Nucleic acid  
 (C) STRANDEDNESS: Double  
 (D) TOPOLOGY: Linear

5 (ii) MOLECULE TYPE: cDNA  
 (ix) FEATURE  
 (C) IDENTIFICATION METHOD: S  
 (xi) SEQUENCE DESCRIPTION: SEQ ID NO:25:

10 GGCCCTGCTGC TGGTCACCTA CCTGCTCCCT CTGCTGGTCA TCCTCCTGTC TTACGTCCGG 60  
 GTGTCAAGTGA AGCTCCGCAA CCGCGTGGTG CCGGGCTGCG TGACCCAGAG CCAGGCCGAC 120  
 TGGGACCGCG CTCGGCGCCG GCGCACCTTC TGCTTGCTGG TGGTGGTCGT GGTGGTG 177

## (2) INFORMATION FOR SEQ ID NO:26:

15 (i) SEQUENCE CHARACTERISTICS:  
 (A) LENGTH: 1110  
 (B) TYPE: Nucleic acid  
 (C) STRANDEDNESS: Double  
 (D) TOPOLOGY: Linear

20 (ii) MOLECULE TYPE: cDNA  
 (ix) FEATURE  
 (C) IDENTIFICATION METHOD: S  
 (xi) SEQUENCE DESCRIPTION: SEQ ID NO:26:

25 ATGGCCTCAT CGACCACTCG GGGCCCCAGG GTTCTGACT TATTTCTGG GCTGCCGCCG 60  
 GCGGTCACAA CTCCCGCCAA CCAGAGCGCA GAGGCTCGG CGGGCAACGG GTCGGTGGCT 120  
 GGCGCGGACG CTCCAGCCGT CACGCCCTTC CAGAGCCTGC AGGTGGTGCA TCAGCTGAAG 180  
 GGGCTGATCG TGCTGCTCTA CAGCGTCGTG GTGGTCGTGG GGCTGGTGGG CAACTGCCTG 240  
 CTGGTGCTGG TGATCGCGCG GGTGCGCCGG CTGCACAACG TGACGAACTT CCTCATCGGC 300  
 30 AACCTGGCCT TGTCCGACGT GCTCATGTGC ACCGCCTGCG TGCCGCTCAC GCTGGCCTAT 360  
 GCCTTCGAGC CACGCGGCTG GGTGTTCCGG GCGGGCCTGT GCCACCTGGT CTTCTTCCTG 420  
 CAGCCGGTCA CCGTCTATGT GTCGGTGTTC ACGCTACCA CCATCGCAGT GGACCGCTAC 480  
 GTCGTGCTGG TGCACCCGCT GAGGCGGCGC ATCTCGCTGC GCCTCAGCGC CTACGCTGTG 540  
 CTGGCCATCT GGGCGCTGTC CGCGGTGCTG GCGCTGCCCG CCGCCGTGCA CACCTATCAC 600  
 35 GTGGAGCTCA AGCCGCACGA CGTGCGCCTC TGCGAGGAGT TCTGGGGCTC CCAGGAGCGC 660  
 CAGCGCCAGC TCTACGCCTG GGGGCTGCTG CTGGTCACCT ACCTGCTCCC TCTGCTGGTC 720



ATCCTCCTGT CTTACGTCCG GGTGTCAGTG AAGCTCCGCA ACCGCGTGGT GCCGGGCTGC 780  
 GTGACCCAGA GCCAGGCCGA CTGGGACCGC GCTCGGCGCC GGCGCACCTT CTGCTTGCTG 840  
 GTGGTGGTCG TGGTGGTGT CGCCGTCTGC TGGCTGCCGC TGCACGTCTT CAACCTGCTG 900  
 CGGGACCTCG ACCCCCACGC CATCGACCCT TACGCCTTTG GGCTGGTGCA GCTGCTCTGC 960  
 5 CACTGGCTCG CCATGAGTTC GGCCTGCTAC AACCCTTCA TCTACGCCTG GCTGCACGAC 1020  
 AGCTTCGCG AGGAGCTGCG CAAACTGTTG GTCGCTTGGC CCCGCAAGAT AGCCCCCAT 1080  
 GGCCAGAATA TGACCGTCAG CGTGGTCATC 1110

## (2) INFORMATION FOR SEQ ID NO:27:

## 10 (i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 618  
 (B) TYPE: Nucleic acid  
 (C) STRANDEDNESS: Double  
 (D) TOPOLOGY: Linear

## 15 (ii) MOLECULE TYPE: cDNA

## (ix) FEATURE

(C) IDENTIFICATION METHOD: S

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:27:

20 CTGGTGCTGG TGATCGCGCG GGTGCGCCGG CTGTACAACG TGACGAATTT CCTCATCGGC 60  
 AACCTGGCCT TGTCCGACGT GCTCATGTGC ACCGCCTGCG TGCCGCTCAC GCTGGCCTAT 120  
 GCCTTCGAGC CACGCGGCTG GGTGTTCCGC GGCGGCCTGT GCCACCTGGT CTTCTTCCTG 180  
 CAGGCGGTCA CCGTCTATGT GTCGGTGTTT ACGCTCACCA CCATCGCAGT GGACCGCTAC 240  
 GTCGTGCTGG TGCACCCGCT GAGGCGGCGC ATCTCGCTGC GCCTCAGCGC CTACGCTGTG 300  
 25 CTGGCCATCT GGGTGCTGTC CGCGGTGCTG GCGCTGCCCC CCGCCGTGCA CACCTATCAC 360  
 GTGGAGCTCA AGCCGCACGA CGTGCGCCTC TGCGAGGAGT TCTGGGGCTC CCAGGAGCGC 420  
 CAGCGCCAGC TCTACGCCTG GGGGCTGCTG CTGGTCACCT ACCTGCTCCC TCTGCTGGTC 480  
 ATCCTCCTGT CTTACGCCCC GGTGTCAGTG AAGCTCCGCA ACCGCGTGGT GCCGGGCCGC 540  
 GTGACCCAGA GCCAGGCCGA CTGGGACCGC GCTCGGCGCC GGCGCACCTT CTGCTTGCTG 600  
 30 GTGGTGGTCG TGGTGGTG 618

## (2) INFORMATION FOR SEQ ID NO:28:

## (i) SEQUENCE CHARACTERISTICS:

35 (A) LENGTH: 378  
 (B) TYPE: Nucleic acid  
 (C) STRANDEDNESS: Double

(D) TOPOLOGY: Linear

(ii) MOLECULE TYPE: cDNA

(ix) FEATURE

(C) IDENTIFICATION METHOD: S

5 (xi) SEQUENCE DESCRIPTION: SEQ ID NO:28:

```

GTGGTTCTGG TGCACCCGCT ACGTCGGCGC ATTTCACTGA GGCTCAGCGC CTACGCGGTG   60
CTGGGCATCT GGGCTCTATC TGCAGTGCTG GCGTGCCGG CCGCGGTGCA CACCTACCAT   120
GTGGAGCTCA AGCCCCACGA CGTGAGCCTC TGCAGGAGT TCTGGGGCTC GCAGGAGCGC   180
10 CAACGCCAGA TCTACGCCTG GGGGCTGCTT CTGGGCACCT ATTTGCTCCC CCTGCTGGCC   240
ATCCTCCTGT CTTACGTACG GGTGTCAGTG AAGCTGAGGA ACCGCGTGGT GCCTGGCAGC   300
GTGACCCAGA GTCAAGCTGA CTGGGACCGA GCGCGTCGCC GCCGCACTTT CTGTCTGCTG   360
GTGGTGGTGG TGGTAGTG                                     378

```

15 (2) INFORMATION FOR SEQ ID NO:29:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 25

(B) TYPE: Nucleic acid

(C) STRANDEDNESS: Single

20 (D) TOPOLOGY: Linear

(ii) MOLECULE TYPE: Other nucleic acid  
Synthetic DNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:29:

25 CGTGGSCMTS STGGGCAACN YCCTG 25

(2) INFORMATION FOR SEQ ID NO:30:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 27

30 (B) TYPE: Nucleic acid

(C) STRANDEDNESS: Single

(D) TOPOLOGY: Linear

(ii) MOLECULE TYPE: Other nucleic acid  
Synthetic DNA

35 (xi) SEQUENCE DESCRIPTION: SEQ ID NO:30:

GTNGWRRGGC ANCCAGCAGA KGGCAAA 27

(2) INFORMATION FOR SEQ ID NO:31:

(i) SEQUENCE CHARACTERISTICS:

5 (A) LENGTH: 27  
 (B) TYPE: Nucleic acid  
 (C) STRANDEDNESS: Single  
 (D) TOPOLOGY: Linear  
 (ii) MOLECULE TYPE: Other nucleic acid  
 10 Synthetic DNA  
 (xi) SEQUENCE DESCRIPTION: SEQ ID NO:31:

CTGTGYGYSA TYGCNNTKGA YMGSTAC 27

15 (2) INFORMATION FOR SEQ ID NO:32:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 29  
 (B) TYPE: Nucleic acid  
 (C) STRANDEDNESS: Single  
 20 (D) TOPOLOGY: Linear  
 (ii) MOLECULE TYPE: Other nucleic acid  
 Synthetic DNA  
 (xi) SEQUENCE DESCRIPTION: SEQ ID NO:32:

25 AKGWAGWAGG GCAGCCAGCA GANSRYGAA 29

(2) INFORMATION FOR SEQ ID NO:33:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 24  
 30 (B) TYPE: Nucleic acid  
 (C) STRANDEDNESS: Single  
 (D) TOPOLOGY: Linear  
 (ii) MOLECULE TYPE: Other nucleic acid  
 Synthetic DNA  
 35 (xi) SEQUENCE DESCRIPTION: SEQ ID NO:33:

CTGACTTATT TTCTGGGCTG CCGC

24

## (2) INFORMATION FOR SEQ ID NO:34:

## (i) SEQUENCE CHARACTERISTICS:

- 5 (A) LENGTH: 24  
 (B) TYPE: Nucleic acid  
 (C) STRANDEDNESS: Single  
 (D) TOPOLOGY: Linear  
 (ii) MOLECULE TYPE: Other nucleic acid  
 10 Synthetic DNA  
 (xi) SEQUENCE DESCRIPTION: SEQ ID NO:34:

AACACCGACA CATAGACGGT GACC

24

## 15 (2) INFORMATION FOR SEQ ID NO:35:

## (i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 20  
 (B) TYPE: Nucleic acid  
 (C) STRANDEDNESS: Single  
 20 (D) TOPOLOGY: Linear  
 (ii) MOLECULE TYPE: Other nucleic acid  
 Synthetic DNA  
 (xi) SEQUENCE DESCRIPTION: SEQ ID NO:35:

25 GCICAYCARC AYTGYATGGA 20

## (2) INFORMATION FOR SEQ ID NO:36:

## (i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 26  
 30 (B) TYPE: Nucleic acid  
 (C) STRANDEDNESS: Single  
 (D) TOPOLOGY: Linear  
 (ii) MOLECULE TYPE: Other nucleic acid  
 Synthetic DNA  
 35 (xi) SEQUENCE DESCRIPTION: SEQ ID NO:36:

CCIACGGGIC KDATGCCICK GCCIGC 26

(2) INFORMATION FOR SEQ ID NO:37:

(i) SEQUENCE CHARACTERISTICS:

- 5 (A) LENGTH: 26  
 (B) TYPE: Nucleic acid  
 (C) STRANDEDNESS: Single  
 (D) TOPOLOGY: Linear  
 (ii) MOLECULE TYPE: Other nucleic acid  
 10 Synthetic DNA  
 (xi) SEQUENCE DESCRIPTION: SEQ ID NO:37:

ACGGGCKDA TGCCICKGCC IGCRTA 26

15 (2) INFORMATION FOR SEQ ID NO:38:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 20  
 (B) TYPE: Nucleic acid  
 (C) STRANDEDNESS: Single  
 20 (D) TOPOLOGY: Linear  
 (ii) MOLECULE TYPE: Other nucleic acid  
 Synthetic DNA  
 (xi) SEQUENCE DESCRIPTION: SEQ ID NO:38:

25 CCGGCGTACC AGGCAGGGTT 20

(2) INFORMATION FOR SEQ ID NO:39:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 28  
 30 (B) TYPE: Nucleic acid  
 (C) STRANDEDNESS: Single  
 (D) TOPOLOGY: Linear  
 (ii) MOLECULE TYPE: Other nucleic acid  
 Synthetic DNA  
 35 (xi) SEQUENCE DESCRIPTION: SEQ ID NO:39:

AGGCAGGGTT GATGTCGGGG GTGCGGAT 28

(2) INFORMATION FOR SEQ ID NO:40:

(i) SEQUENCE CHARACTERISTICS:

- 5 (A) LENGTH: 27  
 (B) TYPE: Nucleic acid  
 (C) STRANDEDNESS: Single  
 (D) TOPOLOGY: Linear  
 (ii) MOLECULE TYPE: Other nucleic acid  
 10 Synthetic DNA  
 (xi) SEQUENCE DESCRIPTION: SEQ ID NO:40:

CTGCCAGCAG AGCCCACCAG CACTCCA 27

15 (2) INFORMATION FOR SEQ ID NO:41:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 27  
 (B) TYPE: Nucleic acid  
 (C) STRANDEDNESS: Single  
 20 (D) TOPOLOGY: Linear  
 (ii) MOLECULE TYPE: Other nucleic acid  
 Synthetic DNA  
 (xi) SEQUENCE DESCRIPTION: SEQ ID NO:41:

25 GTGGGGGCCT GGCTCCTCTG CCTGCTG 27

(2) INFORMATION FOR SEQ ID NO:42:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 32  
 30 (B) TYPE: Nucleic acid  
 (C) STRANDEDNESS: Single  
 (D) TOPOLOGY: Linear  
 (ii) MOLECULE TYPE: Other nucleic acid  
 Synthetic DNA  
 35 (xi) SEQUENCE DESCRIPTION: SEQ ID NO:42:

GTGTCGACGA ATGAAGGCGG TGGGGGCCTG GC 32

(2) INFORMATION FOR SEQ ID NO:43:

(i) SEQUENCE CHARACTERISTICS:

5 (A) LENGTH: 24  
 (B) TYPE: Nucleic acid  
 (C) STRANDEDNESS: Single  
 (D) TOPOLOGY: Linear  
 (ii) MOLECULE TYPE: Other nucleic acid  
 10 Synthetic DNA  
 (xi) SEQUENCE DESCRIPTION: SEQ ID NO:43:

AGGCTCCCGC TGTTATTCCT GGAC 24

15 (2) INFORMATION FOR SEQ ID NO:44:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 98  
 (B) TYPE: Amino acid  
 (C) TOPOLOGY: Linear  
 20 (ii) MOLECULE TYPE: Peptide  
 (xi) SEQUENCE DESCRIPTION: SEQ ID NO:44:

Met Lys Ala Val Gly Ala Trp Leu Leu Cys Leu Leu Leu Gly Leu  
 1 5 10 15  
 25 Ala Leu Gln Gly Ala Ala Ser Arg Ala His Gln His Ser Met Glu Ile  
 20 25 30  
 Arg Thr Pro Asp Ile Asn Pro Ala Trp Tyr Ala Gly Arg Gly Ile Arg  
 35 40 45  
 Pro Val Gly Arg Phe Gly Arg Arg Arg Ala Ala Leu Gly Asp Gly Pro  
 30 50 55 60  
 Arg Pro Gly Pro Arg Arg Val Pro Ala Cys Phe Arg Leu Glu Gly Gly  
 65 70 75 80  
 Ala Glu Pro Ser Arg Ala Leu Pro Gly Arg Leu Thr Ala Gln Leu Val  
 85 90 95  
 35 Gln Glu

## (2) INFORMATION FOR SEQ ID NO:45:

## (i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 83

(B) TYPE: Amino acid

5 (C) TOPOLOGY: Linear

(ii) MOLECULE TYPE: Peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:45:

Met Ala Leu Lys Thr Trp Leu Leu Cys Leu Leu Leu Leu Ser Leu Val  
 10 1 5 10 15  
 Leu Pro Gly Ala Ser Ser Arg Ala His Gln His Ser Met Glu Thr Arg  
 20 25 30  
 Thr Pro Asp Ile Asn Pro Ala Trp Tyr Thr Gly Arg Gly Ile Arg Pro  
 35 40 45  
 15 Val Gly Arg Phe Gly Arg Arg Arg Ala Thr Pro Arg Asp Val Thr Gly  
 50 55 60  
 Leu Gly Gln Leu Ser Cys Leu Pro Leu Asp Gly Arg Thr Lys Phe Ser  
 65 70 75 80  
 Gln Arg Gly

20

## (2) INFORMATION FOR SEQ ID NO:46:

## (i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 249

(B) TYPE: Nucleic acid

25 (C) STRANDEDNESS: Double

(D) TOPOLOGY: Linear

(ii) MOLECULE TYPE: cDNA

(ix) FEATURE

(C) IDENTIFICATION METHOD: S

30 (xi) SEQUENCE DESCRIPTION: SEQ ID NO:46:

ATGGCCCTGA AGACGTGGCT TCTGTGCTTG CTGCTGCTAA GCTTGGTCCT CCCAGGGGCT 60  
 TCCAGCCGAG CCCACCAGCA CTCCATGGAG ACAAGAACCC CTGATATCAA TCCTGCCTGG 120  
 TACACGGGCC GCGGGATCAG GCCTGTGGGC CGCTTCGGCA GGAGAAGGGC AACCCCGAGG 180  
 35 GATGTCACTG GACTTGGCCA ACTCAGCTGC CTCCCACTGG ATGGACGCAC CAAGTTCTCT 240  
 CAGCGTGGA 249



## (2) INFORMATION FOR SEQ ID NO:47:

## (i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 31

(B) TYPE: Amino acid

5 (C) TOPOLOGY: Linear

(ii) MOLECULE TYPE: Peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:47:

Ser Arg Ala His Gln His Ser Met Glu Thr Arg Thr Pro Asp Ile Asn  
 10 1 5 10 15  
 Pro Ala Trp Tyr Thr Gly Arg Gly Ile Arg Pro Val Gly Arg Phe  
 20 25 30

## (2) INFORMATION FOR SEQ ID NO:48:

## 15 (i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 32

(B) TYPE: Amino acid

(C) TOPOLOGY: Linear

(ii) MOLECULE TYPE: Peptide

20 (xi) SEQUENCE DESCRIPTION: SEQ ID NO:48:

Ser Arg Ala His Gln His Ser Met Glu Thr Arg Thr Pro Asp Ile Asn  
 1 5 10 15  
 Pro Ala Trp Tyr Thr Gly Arg Gly Ile Arg Pro Val Gly Arg Phe Gly  
 25 20 25 30

## (2) INFORMATION FOR SEQ ID NO:49:

## (i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 33

30 (B) TYPE: Amino acid

(C) TOPOLOGY: Linear

(ii) MOLECULE TYPE: Peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:49:

35 Ser Arg Ala His Gln His Ser Met Glu Thr Arg Thr Pro Asp Ile Asn  
 1 5 10 15

Pro Ala Trp Tyr Thr Gly Arg Gly Ile Arg Pro Val Gly Arg Phe Gly  
 20 25 30

Arg

5 (2) INFORMATION FOR SEQ ID NO:50:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 20

(B) TYPE: Amino acid

(C) TOPOLOGY: Linear

10 (ii) MOLECULE TYPE: Peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:50:

Thr Pro Asp Ile Asn Pro Ala Trp Tyr Thr Gly Arg Gly Ile Arg Pro  
 1 5 10 15  
 15 Val Gly Arg Phe  
 20

(2) INFORMATION FOR SEQ ID NO:51:

(i) SEQUENCE CHARACTERISTICS:

20 (A) LENGTH: 21

(B) TYPE: Amino acid

(C) TOPOLOGY: Linear

(ii) MOLECULE TYPE: Peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:51:

25

Thr Pro Asp Ile Asn Pro Ala Trp Tyr Thr Gly Arg Gly Ile Arg Pro  
 1 5 10 15  
 Val Gly Arg Phe Gly  
 20

30

(2) INFORMATION FOR SEQ ID NO:52:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 22

(B) TYPE: Amino acid

35 (C) TOPOLOGY: Linear

(ii) MOLECULE TYPE: Peptide

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:52:

Thr Pro Asp Ile Asn Pro Ala Trp Tyr Thr Gly Arg Gly Ile Arg Pro  
 1 5 10 15  
 5 Val Gly Arg Phe Gly Arg  
 20

## (2) INFORMATION FOR SEQ ID NO:53:

## (i) SEQUENCE CHARACTERISTICS:

10 (A) LENGTH: 93  
 (B) TYPE: Nucleic acid  
 (C) STRANDEDNESS: Double  
 (D) TOPOLOGY: Linear

(ii) MOLECULE TYPE: cDNA

15 (ix) FEATURE

(C) IDENTIFICATION METHOD: S

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:53:

AGCCGAGCCC ACCAGCACTC CATGGAGACA AGAACCCCTG ATATCAATCC TGCCTGGTAC 60  
 20 ACGGGCCGCG GGATCAGGCC TGTGGGCCGC TTC 93

## (2) INFORMATION FOR SEQ ID NO:54:

## (i) SEQUENCE CHARACTERISTICS:

25 (A) LENGTH: 96  
 (B) TYPE: Nucleic acid  
 (C) STRANDEDNESS: Double  
 (D) TOPOLOGY: Linear

(ii) MOLECULE TYPE: cDNA

(ix) FEATURE

30 (C) IDENTIFICATION METHOD: S

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:54:

AGCCGAGCCC ACCAGCACTC CATGGAGACA AGAACCCCTG ATATCAATCC TGCCTGGTAC 60  
 ACGGGCCGCG GGATCAGGCC TGTGGGCCGC TTCGGC 96  
 35

## (2) INFORMATION FOR SEQ ID NO:55:

## (i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 99  
 (B) TYPE: Nucleic acid  
 (C) STRANDEDNESS: Double  
 (D) TOPOLOGY: Linear

5

(ii) MOLECULE TYPE: cDNA

(ix) FEATURE

(C) IDENTIFICATION METHOD: S

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:55:

10

AGCCGAGCCC ACCAGCACTC CATGGAGACA AGAACCCCTG ATATCAATCC TGCCTGGTAC 60  
 ACGGGCCGCG GGATCAGGCC TGTGGGCCGC TTCGGCAGG 99

## (2) INFORMATION FOR SEQ ID NO:56:

15

## (i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 60  
 (B) TYPE: Nucleic acid  
 (C) STRANDEDNESS: Double  
 (D) TOPOLOGY: Linear

20

(ii) MOLECULE TYPE: cDNA

(ix) FEATURE

(C) IDENTIFICATION METHOD: S

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:56:

25

ACCCCTGATA TCAATCCTGC CTGGTACACG GGCCGCGGGA TCAGGCCTGT GGGCCGCTTC 60

## (2) INFORMATION FOR SEQ ID NO:57:

## (i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 63  
 (B) TYPE: Nucleic acid  
 (C) STRANDEDNESS: Double  
 (D) TOPOLOGY: Linear

30

(ii) MOLECULE TYPE: cDNA

(ix) FEATURE

35

(C) IDENTIFICATION METHOD: S

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:57:

ACCCCTGATA TCAATCCTGC CTGGTACACG GGCCGCGGGA TCAGGCCTGT GGGCCGCTTC 60  
GGC 63

(2) INFORMATION FOR SEQ ID NO:58:

5 (i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 66  
(B) TYPE: Nucleic acid  
(C) STRANDEDNESS: Double  
(D) TOPOLOGY: Linear

10 (ii) MOLECULE TYPE: cDNA

(ix) FEATURE

(C) IDENTIFICATION METHOD: S

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:58:

15 ACCCCTGATA TCAATCCTGC CTGGTACACG GGCCGCGGGA TCAGGCCTGT GGGCCGCTTC 60  
GGCAGG 66

(2) INFORMATION FOR SEQ ID NO:59:

(i) SEQUENCE CHARACTERISTICS:

20 (A) LENGTH: 87  
(B) TYPE: Amino acid  
(C) TOPOLOGY: Linear

(ii) MOLECULE TYPE: Peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:59:

25

Met Lys Val Leu Arg Ala Trp Leu Leu Cys Leu Leu Met Leu Gly Leu

1 5 10 15

Ala Leu Arg Gly Ala Ala Ser Arg Thr His Arg His Ser Met Glu Ile

20 25 30

30 Arg Thr Pro Asp Ile Asn Pro Ala Trp Tyr Ala Ser Arg Gly Ile Arg

35 40 45

Pro Val Gly Arg Phe Gly Arg Arg Arg Ala Thr Leu Gly Asp Val Pro

50 55 60

Lys Pro Gly Leu Arg Pro Arg Leu Thr Cys Phe Pro Leu Glu Gly Gly

35 65 70 75 80

Ala Met Ser Ser Gln Asp Gly

85

## (2) INFORMATION FOR SEQ ID NO:60:

## (i) SEQUENCE CHARACTERISTICS:

5 (A) LENGTH: 261  
 (B) TYPE: Nucleic acid  
 (C) STRANDEDNESS: Double  
 (D) TOPOLOGY: Linear

(ii) MOLECULE TYPE: cDNA

10 (ix) FEATURE

(C) IDENTIFICATION METHOD: S

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:60:

ATGAAGGTGC TGAGGGCCTG GCTCCTGTGC CTGCTGATGC TGGGCCTGGC CCTGCGGGGA 60  
 15 GCTGCAAGTC GTACCCATCG GCACTCCATG GAGATCCGCA CCCCTGACAT CAATCCTGCC 120  
 TGGTACGCCA GTCGCGGGAT CAGGCCTGTG GGCCGCTTCG GTCGGAGGAG GGCAACCCTG 180  
 GGGGACGTCC CCAAGCCTGG CCTGCGACCC CGGCTGACCT GCTTCCCCCT GGAAGGCGGT 240  
 GCTATGTCGT CCCAGGATGG C 261

## 20 (2) INFORMATION FOR SEQ ID NO:61:

## (i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 31  
 (B) TYPE: Amino acid  
 (C) TOPOLOGY: Linear

25 (ii) MOLECULE TYPE: Peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:61:

Ser Arg Thr His Arg His Ser Met Glu Ile Arg Thr Pro Asp Ile Asn  
 1 5 10 15  
 30 Pro Ala Trp Tyr Ala Ser Arg Gly Ile Arg Pro Val Gly Arg Phe  
 20 25 30

## (2) INFORMATION FOR SEQ ID NO:62:

## (i) SEQUENCE CHARACTERISTICS:

35 (A) LENGTH: 32  
 (B) TYPE: Amino acid

(C) TOPOLOGY: Linear  
(ii) MOLECULE TYPE: Peptide  
(xi) SEQUENCE DESCRIPTION: SEQ ID NO:62:

5 Ser Arg Thr His Arg His Ser Met Glu Ile Arg Thr Pro Asp Ile Asn  
1 5 10 15  
Pro Ala Trp Tyr Ala Ser Arg Gly Ile Arg Pro Val Gly Arg Phe Gly  
20 25 30

10 (2) INFORMATION FOR SEQ ID NO:63:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 33

(B) TYPE: Amino acid

(C) TOPOLOGY: Linear

15 (ii) MOLECULE TYPE: Peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:63:

Ser Arg Thr His Arg His Ser Met Glu Ile Arg Thr Pro Asp Ile Asn  
1 5 10 15  
20 Pro Ala Trp Tyr Ala Ser Arg Gly Ile Arg Pro Val Gly Arg Phe Gly  
20 25 30  
Arg

(2) INFORMATION FOR SEQ ID NO:64:

25 (i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 20

(B) TYPE: Amino acid

(C) TOPOLOGY: Linear

(ii) MOLECULE TYPE: Peptide

30 (xi) SEQUENCE DESCRIPTION: SEQ ID NO:64:

Thr Pro Asp Ile Asn Pro Ala Trp Tyr Ala Ser Arg Gly Ile Arg Pro  
1 5 10 15  
Val Gly Arg Phe  
35 20

## (2) INFORMATION FOR SEQ ID NO:65:

## (i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 21

(B) TYPE: Amino acid

5 (C) TOPOLOGY: Linear

(ii) MOLECULE TYPE: Peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:65:

Thr Pro Asp Ile Asn Pro Ala Trp Tyr Ala Ser Arg Gly Ile Arg Pro  
 10 1 5 10 15  
 Val Gly Arg Phe Gly  
 20

## (2) INFORMATION FOR SEQ ID NO:66:

## 15 (i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 22

(B) TYPE: Amino acid

(C) TOPOLOGY: Linear

(ii) MOLECULE TYPE: Peptide

20 (xi) SEQUENCE DESCRIPTION: SEQ ID NO:66:

Thr Pro Asp Ile Asn Pro Ala Trp Tyr Ala Ser Arg Gly Ile Arg Pro  
 1 5 10 15  
 Val Gly Arg Phe Gly Arg  
 25 20

## (2) INFORMATION FOR SEQ ID NO:67:

## (i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 93

30 (B) TYPE: Nucleic acid

(C) STRANDEDNESS: Double

(D) TOPOLOGY: Linear

(ii) MOLECULE TYPE: cDNA

(ix) FEATURE

35 (C) IDENTIFICATION METHOD: S

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:67:



AGTCGTACCC ATCGGCACTC CATGGAGATC CGCACCCCTG ACATCAATCC TGCCTGGTAC 60  
GCCAGTCGCG GGATCAGGCC TGTGGGCCGC TTC 93

## (2) INFORMATION FOR SEQ ID NO:68:

## 5 (i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 96  
(B) TYPE: Nucleic acid  
(C) STRANDEDNESS: Double  
(D) TOPOLOGY: Linear

## 10 (ii) MOLECULE TYPE: cDNA

## (ix) FEATURE

(C) IDENTIFICATION METHOD: S

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:68:

15 AGTCGTACCC ATCGGCACTC CATGGAGATC CGCACCCCTG ACATCAATCC TGCCTGGTAC 60  
GCCAGTCGCG GGATCAGGCC TGTGGGCCGC TTCGGT 96

## (2) INFORMATION FOR SEQ ID NO:69:

## (i) SEQUENCE CHARACTERISTICS:

20 (A) LENGTH: 99  
(B) TYPE: Nucleic acid  
(C) STRANDEDNESS: Double  
(D) TOPOLOGY: Linear

## (ii) MOLECULE TYPE: cDNA

## 25 (ix) FEATURE

(C) IDENTIFICATION METHOD: S

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:69:

AGTCGTACCC ATCGGCACTC CATGGAGATC CGCACCCCTG ACATCAATCC TGCCTGGTAC 60  
30 GCCAGTCGCG GGATCAGGCC TGTGGGCCGC TTCGGTCGG 99

## (2) INFORMATION FOR SEQ ID NO:70:

## (i) SEQUENCE CHARACTERISTICS:

35 (A) LENGTH: 60  
(B) TYPE: Nucleic acid  
(C) STRANDEDNESS: Double

(D) TOPOLOGY: Linear

(ii) MOLECULE TYPE: cDNA

(ix) FEATURE

(C) IDENTIFICATION METHOD: S

5 (xi) SEQUENCE DESCRIPTION: SEQ ID NO:70:

ACCCCTGACA TCAATCCTGC CTGGTACGCC AGTCGCGGGA TCAGGCCTGT GGGCCGCTTC 60

(2) INFORMATION FOR SEQ ID NO:71:

10 (i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 63

(B) TYPE: Nucleic acid

(C) STRANDEDNESS: Double

(D) TOPOLOGY: Linear

15 (ii) MOLECULE TYPE: cDNA

(ix) FEATURE

(C) IDENTIFICATION METHOD: S

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:71:

20 ACCCCTGACA TCAATCCTGC CTGGTACGCC AGTCGCGGGA TCAGGCCTGT GGGCCGCTTC 60  
GGT 63

(2) INFORMATION FOR SEQ ID NO:72:

(i) SEQUENCE CHARACTERISTICS:

25 (A) LENGTH: 66

(B) TYPE: Nucleic acid

(C) STRANDEDNESS: Double

(D) TOPOLOGY: Linear

(ii) MOLECULE TYPE: cDNA

30 (ix) FEATURE

(C) IDENTIFICATION METHOD: S

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:72:

35 ACCCCTGACA TCAATCCTGC CTGGTACGCC AGTCGCGGGA TCAGGCCTGT GGGCCGCTTC 60  
GGTCGG 66

5  
10

## 15

20

25

30

## 35

(D) TOPOLOGY: Linear  
(ii) MOLECULE TYPE: Other nucleic acid  
Synthetic DNA  
(xi) SEQUENCE DESCRIPTION: SEQ ID NO:75:

5

CARCAYTCCA TGGAGACAAG AACCCC 26

(2) INFORMATION FOR SEQ ID NO:76:

(i) SEQUENCE CHARACTERISTICS:

10 (A) LENGTH: 24  
(B) TYPE: Nucleic acid  
(C) STRANDEDNESS: Single  
(D) TOPOLOGY: Linear  
(ii) MOLECULE TYPE: Other nucleic acid  
15 Synthetic DNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:76:

TACCAGGCAG GATTGATACA GGGG 24

20 (2) INFORMATION FOR SEQ ID NO:77:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 25  
(B) TYPE: Nucleic acid  
(C) STRANDEDNESS: Single  
25 (D) TOPOLOGY: Linear  
(ii) MOLECULE TYPE: Other nucleic acid  
Synthetic DNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:77:

30 GGCATCATCC AGGAAGACGG AGCAT 25

(2) INFORMATION FOR SEQ ID NO:78:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 25  
35 (B) TYPE: Nucleic acid  
(C) STRANDEDNESS: Single

(D) TOPOLOGY: Linear  
(ii) MOLECULE TYPE: Other nucleic acid  
Synthetic DNA  
(xi) SEQUENCE DESCRIPTION: SEQ ID NO:78:

5

AGCAGAGGAG AGGGAGGGTA GAGGA 25

(2) INFORMATION FOR SEQ ID NO:79:

(i) SEQUENCE CHARACTERISTICS:

10 (A) LENGTH: 22  
(B) TYPE: Nucleic acid  
(C) STRANDEDNESS: Single  
(D) TOPOLOGY: Linear  
(ii) MOLECULE TYPE: Other nucleic acid  
15 Synthetic DNA  
(xi) SEQUENCE DESCRIPTION: SEQ ID NO:79:

ACGTGGCTTC TGTGCTTGCT GC 22

20 (2) INFORMATION FOR SEQ ID NO:80:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 25  
(B) TYPE: Nucleic acid  
(C) STRANDEDNESS: Single  
25 (D) TOPOLOGY: Linear  
(ii) MOLECULE TYPE: Other nucleic acid  
Synthetic DNA  
(xi) SEQUENCE DESCRIPTION: SEQ ID NO:80:

30 GCCTGATCCC GCGGCCCGTG TACCA 25

(2) INFORMATION FOR SEQ ID NO:81:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 26  
35 (B) TYPE: Nucleic acid  
(C) STRANDEDNESS: Single

(D) TOPOLOGY: Linear  
(ii) MOLECULE TYPE: Other nucleic acid  
Synthetic DNA  
(xi) SEQUENCE DESCRIPTION: SEQ ID NO:81:

5

TTGCCCTTCT CCTGCCGAAG CGGCCC 26

(2) INFORMATION FOR SEQ ID NO:82:

(i) SEQUENCE CHARACTERISTICS:

10

(A) LENGTH: 27

(B) TYPE: Nucleic acid

(C) STRANDEDNESS: Single

(D) TOPOLOGY: Linear

(ii) MOLECULE TYPE: Other nucleic acid

15

Synthetic DNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:82:

GGCGGGGGCT GCAAGTCGTA CCCATCG 27

20 (2) INFORMATION FOR SEQ ID NO:83:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 27

(B) TYPE: Nucleic acid

(C) STRANDEDNESS: Single

25

(D) TOPOLOGY: Linear

(ii) MOLECULE TYPE: Other nucleic acid

Synthetic DNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:83:

30 CGGCACTCCA TGGAGATCCG CACCGCT 27

(2) INFORMATION FOR SEQ ID NO:84:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 27

35

(B) TYPE: Nucleic acid

(C) STRANDEDNESS: Single

(D) TOPOLOGY: Linear  
(ii) MOLECULE TYPE: Other nucleic acid  
Synthetic DNA  
(xi) SEQUENCE DESCRIPTION: SEQ ID NO:84:

5

CAGGCAGGAT TGATGTCAGG GGTGCGG 27

## (2) INFORMATION FOR SEQ ID NO:85:

## (i) SEQUENCE CHARACTERISTICS:

10

(A) LENGTH: 27

(B) TYPE: Nucleic acid

(C) STRANDEDNESS: Single

(D) TOPOLOGY: Linear

(ii) MOLECULE TYPE: Other nucleic acid

15

Synthetic DNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:85:

CATGGAGTGC CGATGGGTAC GACTTGC 27

## 20 (2) INFORMATION FOR SEQ ID NO:86:

## (i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 27

(B) TYPE: Nucleic acid

(C) STRANDEDNESS: Single

25

(D) TOPOLOGY: Linear

(ii) MOLECULE TYPE: Other nucleic acid

Synthetic DNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:86:

30 GGCCTCCTCG GAGGAGCCAA GGGATGA 27

## (2) INFORMATION FOR SEQ ID NO:87:

## (i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 27

35

(B) TYPE: Nucleic acid

(C) STRANDEDNESS: Single

(D) TOPOLOGY: Linear  
(ii) MOLECULE TYPE: Other nucleic acid  
Synthetic DNA  
(xi) SEQUENCE DESCRIPTION: SEQ ID NO:87:

5

GGGAAAGGAG CCCGAAGGAG AGGAGAG 27

## (2) INFORMATION FOR SEQ ID NO:88:

## (i) SEQUENCE CHARACTERISTICS:

10

(A) LENGTH: 25

(B) TYPE: Nucleic acid

(C) STRANDEDNESS: Single

(D) TOPOLOGY: Linear

(ii) MOLECULE TYPE: Other nucleic acid

15

Synthetic DNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:88:

CCTGCTGGCC ATTCTCCTGT CTTAC 25

## 20 (2) INFORMATION FOR SEQ ID NO:89:

## (i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 25

(B) TYPE: Nucleic acid

(C) STRANDEDNESS: Single

25

(D) TOPOLOGY: Linear

(ii) MOLECULE TYPE: Other nucleic acid

Synthetic DNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:89:

30 GGGTCCAGGT CCCGCAGAAG GTTGA 25

## (2) INFORMATION FOR SEQ ID NO:90:

## (i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 25

35

(B) TYPE: Nucleic acid

(C) STRANDEDNESS: Single



(D) TOPOLOGY: Linear  
(ii) MOLECULE TYPE: Other nucleic acid  
Synthetic DNA  
(xi) SEQUENCE DESCRIPTION: SEQ ID NO:90:

5

GAAGACGGAG CATGGCCCTG AAGAC 25

(2) INFORMATION FOR SEQ ID NO:91:

(i) SEQUENCE CHARACTERISTICS:

10 (A) LENGTH: 25  
(B) TYPE: Nucleic acid  
(C) STRANDEDNESS: Single  
(D) TOPOLOGY: Linear  
(ii) MOLECULE TYPE: Other nucleic acid  
15 Synthetic DNA  
(xi) SEQUENCE DESCRIPTION: SEQ ID NO:91:

GGCAGCTGAG TTGGCCAAGT CCACT 25

20 (2) INFORMATION FOR SEQ ID NO:92:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 15  
(B) TYPE: Amino acid  
(C) TOPOLOGY: Linear  
25 (ii) MOLECULE TYPE: Peptide  
(xi) SEQUENCE DESCRIPTION: SEQ ID NO:92:

Ser Arg Ala His Gln His Ser Met Glu Ile Arg Thr Pro Asp Cys  
1 5 10 15

30

(2) INFORMATION FOR SEQ ID NO:93:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 15  
(B) TYPE: Amino acid  
35 (C) TOPOLOGY: Linear  
(ii) MOLECULE TYPE: Peptide

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:93:

Cys Ala Trp Tyr Ala Gly Arg Gly Ile Arg Pro Val Gly Arg Phe  
 1 5 10 15

5

## (2) INFORMATION FOR SEQ ID NO:94:

## (i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 15

(B) TYPE: Amino acid

10

(C) TOPOLOGY: Linear

(ii) MOLECULE TYPE: Peptide

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:94:

Cys Glu Ile Arg Thr Pro Asp Ile Asn Pro Ala Trp Tyr Ala Gly  
 15 1 5 10 15

## (2) INFORMATION FOR SEQ ID NO:95:

## (i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 24

20

(B) TYPE: Nucleic acid

(C) STRANDEDNESS: Single

(D) TOPOLOGY: Linear

(ii) MOLECULE TYPE: Other nucleic acid  
 Synthetic DNA

25

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:95:

GTTACACAGGT CGACATGACC TCAC 24

## (2) INFORMATION FOR SEQ ID NO:96:

30

## (i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 25

(B) TYPE: Nucleic acid

(C) STRANDEDNESS: Single

(D) TOPOLOGY: Linear

35

(ii) MOLECULE TYPE: Other nucleic acid  
 Synthetic DNA



## CLAIMS

## WHAT IS CLAIMED IS:

1. A polypeptide which comprises an amino acid sequence represented by SEQ ID NO:73 or its substantial equivalent thereto, or its amide or ester, or a salt thereof.
2. The polypeptide as claimed in claim 1, which comprises the amino acid sequence represented by SEQ ID NO:3, SEQ ID NO:4, SEQ ID NO:5, SEQ ID NO:6, SEQ ID NO:7, SEQ ID NO:8, SEQ ID NO:9, SEQ ID NO:10, SEQ ID NO:47, SEQ ID NO:48, SEQ ID NO:49, SEQ ID NO:50, SEQ ID NO:51, SEQ ID NO:52, SEQ ID NO:61, SEQ ID NO:62, SEQ ID NO:63, SEQ ID NO:64, SEQ ID NO:65, or SEQ ID NO:66.
3. The polypeptide as claimed in claim 1, which comprises the amino acid sequence represented by SEQ ID NO:1, SEQ ID NO:44, SEQ ID NO:45, or SEQ ID NO:59.
4. A partial peptide of the polypeptide as claimed in claim 1, or its amide or ester, or a salt thereof.
5. A DNA which comprises a DNA having a nucleotide sequence coding for the polypeptide as claimed in claim 1, or the partial peptide as claimed in claim 4.
6. The DNA as claimed in claim 5 which comprises a nucleotide sequence represented by SEQ ID NO:2, SEQ ID NO:11, SEQ ID NO:12, SEQ ID NO:13, SEQ ID NO:14, SEQ ID NO:15, SEQ ID NO:16, SEQ ID NO:17, SEQ ID NO:18, SEQ ID NO:46, SEQ ID NO:53, SEQ ID NO:54, SEQ ID NO:55, SEQ ID NO:56, SEQ ID NO:57, SEQ ID NO:58, SEQ ID NO:60, SEQ ID NO:67, SEQ ID NO:68, SEQ ID NO:69, SEQ ID NO:70, SEQ ID NO:71, or SEQ ID NO:72.
7. A recombinant vector comprising the DNA as claimed in claim 5.
8. A transformant carrying the DNA as claimed in claim 5 or the recombinant vector as claimed in claim 7.
9. A method for producing the polypeptide as claimed

in claim 1 or the partial peptide as claimed in claim 4, which comprises culturing the transformant as claimed in claim 8.

10. A pharmaceutical composition comprises the polypeptide, its amide or ester as claimed in claim 1, or a pharmaceutically acceptable salt thereof.

11. A pharmaceutical composition containing the partial peptide, its amide ester as claimed in claim 4, or a pharmaceutically acceptable salt thereof.

12. A pharmaceutical composition containing the DNA as claimed in claim 5.

13. The pharmaceutical composition as claimed in claim 10, 11, or 12, which is a pituitary function modulator.

14. The pharmaceutical composition as claimed in claim 10, 11, or 12, which is a central nervous system function modulator.

15. The pharmaceutical composition as claimed in claim 10, 11, or 12, which is a pancreatic function modulator.

16. An antibody against the polypeptide as claimed in claim 1 or against the partial peptide as claimed in claim 4.

17. A screening method for a compound capable of changing the binding activity of the polypeptide as claimed in claim 1 or the partial peptide as claimed in claim 4 with a receptor protein comprising an amino acid sequence represented by SEQ ID NO:21 or its partial peptide or its substantial equivalent thereto, or a salt thereof, which comprises making a comparison between: (i) at least one case where said polypeptide as claimed in claim 1 or the partial peptide as claimed in claim 4 is contacted with a receptor protein comprising an amino acid sequence represented by SEQ ID NO:21 or its partial peptide or its substantial equivalent thereto, or a salt thereof, and (ii) at least one case where said polypeptide as claimed in

claim 1 or the partial peptide as claimed in claim 4 together with a sample to be tested is contacted with the receptor protein comprising an amino acid sequence represented by SEQ ID NO:21 or its partial peptide or its substantial equivalent, or a salt thereof.

18. A kit for screening for a compound capable of changing the binding activity of the polypeptide as claimed in claim 1 or the partial peptide as claimed in claim 4 with a receptor protein comprising an amino acid sequence represented by SEQ ID NO:21 or its partial peptide or its substantial equivalent thereto, or a salt thereof.

19. A compound capable of changing the binding activity of the polypeptide as claimed in claim 1 or the partial peptide as claimed in claim 4 with a receptor protein comprising an amino acid sequence represented by SEQ ID NO:21 or its partial peptide or its substantial equivalent thereto, or a salt thereof, which is obtained by the screening method as claimed in claim 17 or by using the kit for screening as claimed in claim 18.

20. A G protein-coupled receptor protein which recognizes the polypeptide as claimed in claim 1 or the partial peptide as claimed in claim 4 as a ligand, or a salt thereof.

## ABSTRACT

The present invention relates to the ligand polypeptide for the human pituitary- and mouse pancreas-derived G protein-coupled receptor proteins.

The ligand polypeptide or the DNA which codes for the ligand polypeptide can be used for (1) development of medicines such as pituitary function modulators, central nervous system function modulators, and pancreatic function modulators, and (2) development of receptor binding assay systems using the expression of recombinant receptor proteins and screening of pharmaceutical candidate compounds.

In particular, by the receptor binding assay systems utilizing the expression of recombinant G protein-coupled receptor proteins in accordance with the invention, agonists and antagonists of G protein-coupled receptors which are specific to human and other warm-blooded animals can be screened and the agonists or antagonists obtained can be used as therapeutic and prophylactic agents for various diseases.

## 2

•

1



Fig. 2

5'                   9                   18                   27                   36                   45                   54  
 GGC CTG CTG CTG GTC ACC TAC CTG CTC CCT CTG CTG GTC ATC CTC CTG TCT TAC  
 --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- ---  
 Gly Leu Leu Leu Val Thr Tyr Leu Leu Pro Leu Leu Val Ile Leu Leu Ser Tyr  
  
                  63                   72                   81                   90                   99                   108  
 GTC CGG GTG TCA GTG AAG CTC CGC AAC CGC GTG GTG CCG GGC TGC GTG ACC CAG  
 --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- ---  
 Val Arg Val Ser Val Lys Leu Arg Asn Arg Val Val Pro Gly Cys Val Thr Gln  
  
                  117                   126                   135                   144                   153                   162  
 AGC CAG GCC GAC TGG GAC CGC GCT CGG CGC CGG CGC ACC TTC TGC TTG CTG GTG  
 --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- ---  
 Ser Gln Ala Asp Trp Asp Arg Ala Arg Arg Arg Arg Thr Phe Cys Leu Leu Val  
  
                  171                   180                   189                   198  
 GTG GTC GTG GTG GTG TTT GCC ATC TGC TGG TTG CCT TAC TAC 3'  
 --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- ---  
 Val Val Val Val Val Phe Ala Ile Cys Trp Leu Pro Tyr Tyr

Fig. 3

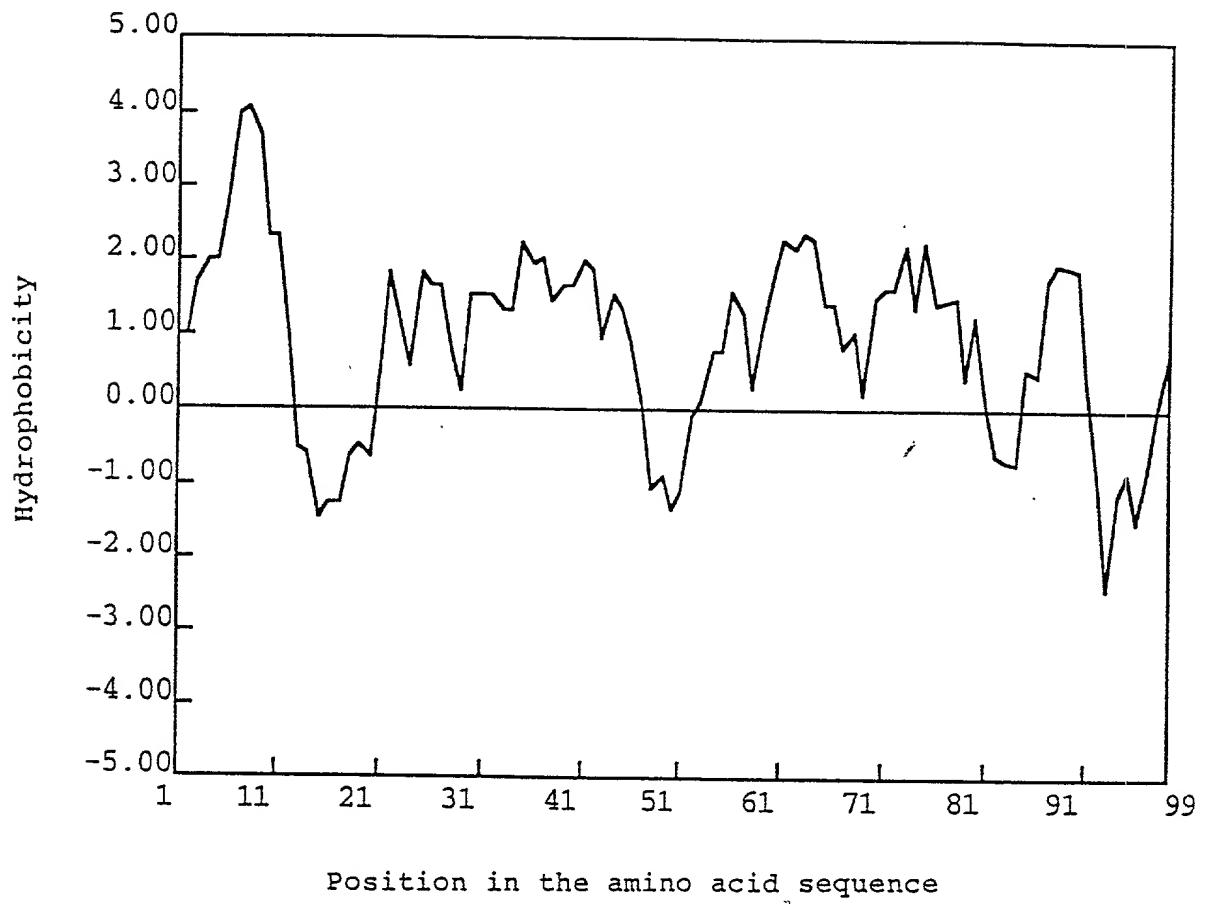
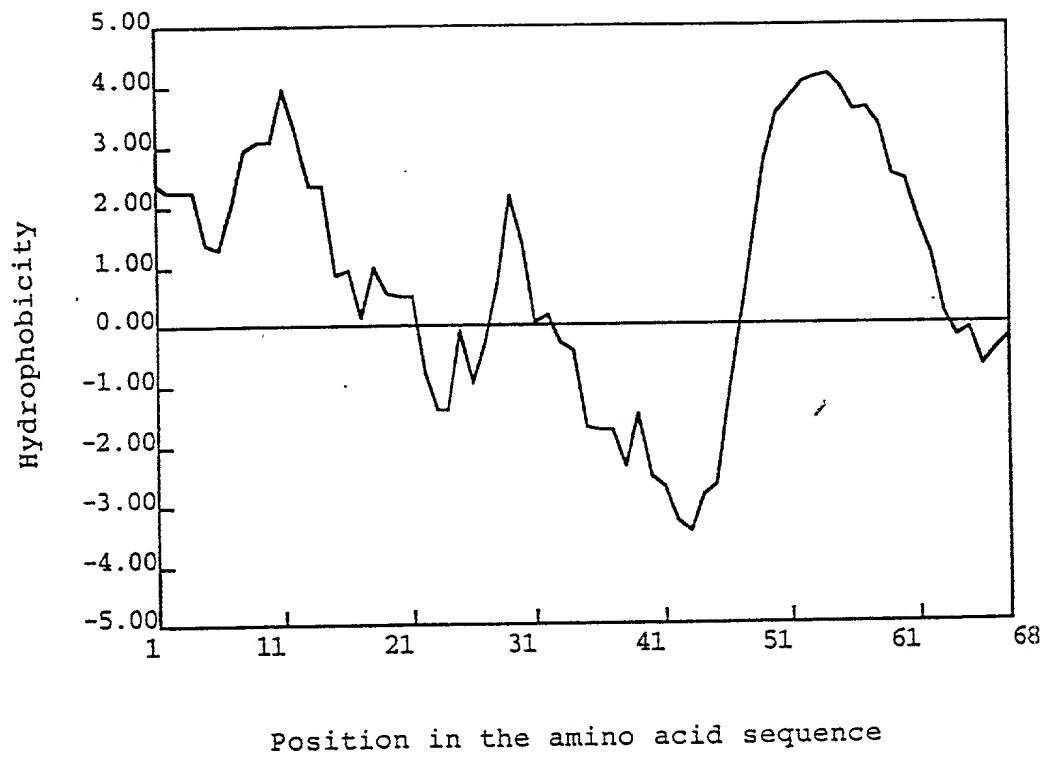


Fig. 4



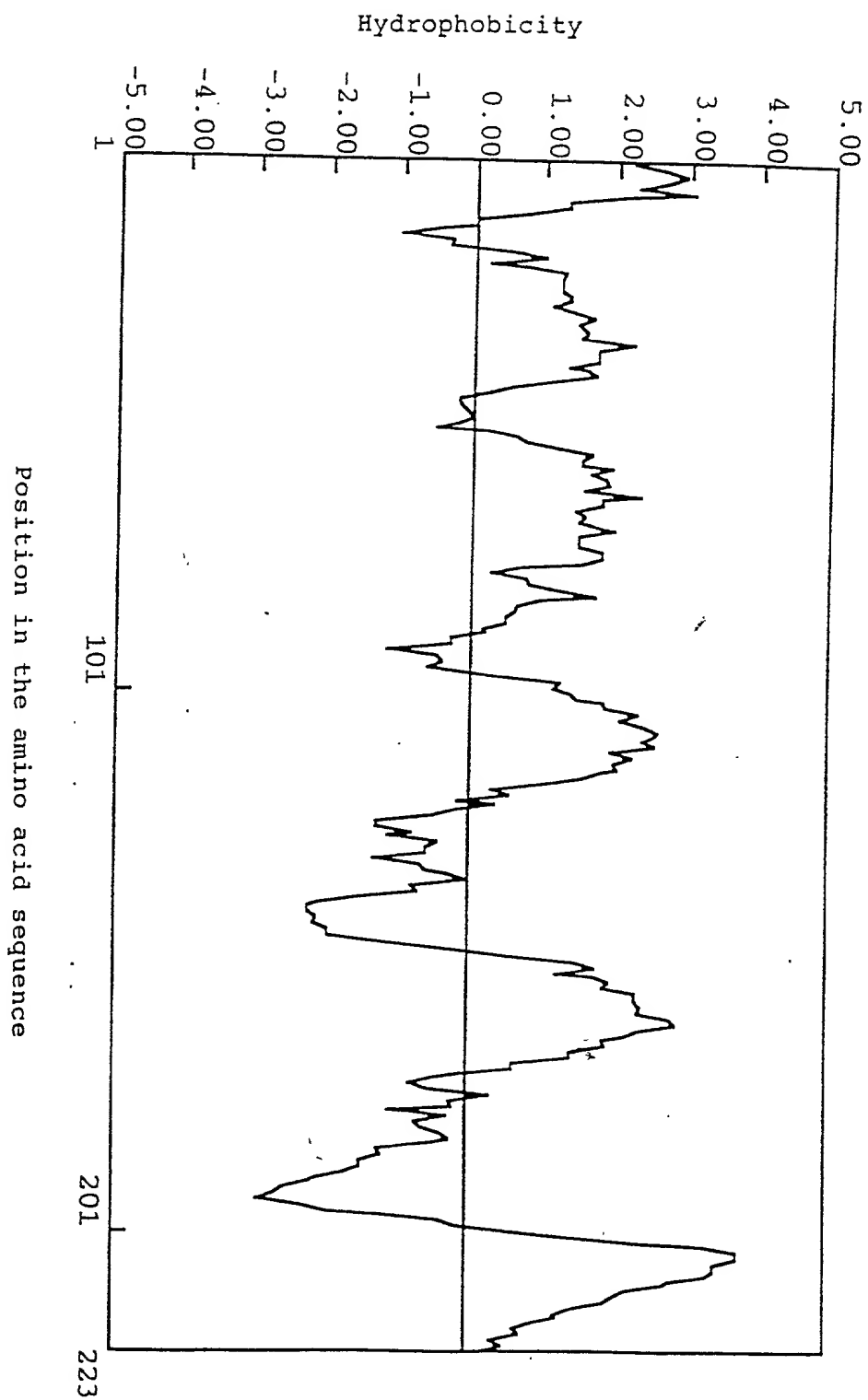
[illegible]

	9			18			27			36			45			54					
5'	GTG	GGC	ATG	GTG	GGC	AAC	ATC	CTG	CTG	GTG	CTG	GTG	ATC	GCG	CGG	GTG	CGC	CGG			
	Val	Gly	Met	Val	Gly	Asn	Ile	Leu	Leu	Val	Leu	Val	Ile	Ala	Arg	Val	Arg	Arg			
	63			72			81			90			99			108					
	CTG	TAC	AAC	GTG	ACG	AAT	TTC	CTC	ATC	GGC	AAC	CTG	GCC	TTG	TCC	GAC	GTG	CTC			
	Leu	Tyr	Asn	Val	Thr	Asn	Phe	Leu	Ile	Gly	Asn	Leu	Ala	Leu	Ser	Asp	Val	Leu			
	117			126			135			144			153			162					
	ATG	TGC	ACC	GCC	TGC	GTG	CCG	CTC	ACG	CTG	GCC	TAT	GCC	TTC	GAG	CCA	CGC	GGC			
	Met	Cys	Thr	Ala	Cys	Val	Pro	Leu	Thr	Leu	Ala	Tyr	Ala	Phe	Glu	Pro	Arg	Gly			
	171			180			189			198			207			216					
	TGG	GTG	TTC	GGC	GGC	GGC	CTG	TGC	CAC	CTG	GTC	TTC	TTC	CTG	CAG	GCG	GTC	ACC			
	Trp	Val	Phe	Gly	Gly	Gly	Leu	Cys	His	Leu	Val	Phe	Phe	Leu	Gln	Ala	Val	Thr			
	225			234			243			252			261			270					
	GTC	TAT	GTG	TGC	GTG	TTC	ACG	CTC	ACC	ACC	ATC	GCA	GTG	GAC	CGC	TAC	GTC	GTG			
	Val	Tyr	Val	Ser	Val	Phe	Thr	Leu	Thr	Thr	Ile	Ala	Val	Asp	Arg	Tyr	Val	Val			
	279			288			297			306			315			324					
	CTG	GTG	CAC	CCG	CTG	AGG	CGG	CGC	ATC	TGC	CTG	CGC	CTC	AGC	GCC	TAC	GCT	GTG			
	Leu	Val	His	Pro	Leu	Arg	Arg	Arg	Ile	Ser	Leu	Arg	Leu	Ser	Ala	Tyr	Ala	Val			
	333			342			351			360			369			378					
	CTG	GCC	ATC	TGG	GTG	CTG	TCC	GCG	GTG	CTG	GCG	CTG	CCC	GCC	GCC	GTG	CAC	ACC			
	Leu	Ala	Ile	Trp	Val	Leu	Ser	Ala	Val	Leu	Ala	Leu	Pro	Ala	Ala	Val	His	Thr			
	387			396			405			414			423			432					
	TAT	CAC	GTG	GAG	CTC	AAG	CCG	CAC	GAC	GTG	CGC	CTC	TGC	GAG	GAG	TTC	TGG	GGC			
	Tyr	His	Val	Glu	Leu	Lys	Pro	His	Asp	Val	Arg	Leu	Cys	Glu	Glu	Phe	Trp	Gly			
	441			450			459			468			477			486					
	TCC	CAG	GAG	CGC	CAG	CGC	CAG	CTC	TAC	GCC	TGG	GGG	CTG	CTG	CTG	GTC	ACC	TAC			
	Ser	Gln	Glu	Arg	Gln	Arg	Gln	Leu	Tyr	Ala	Trp	Gly	Leu	Leu	Leu	Val	Thr	Tyr			
	495			504			513			522			531			540					
	CTG	CTC	CCT	CTG	CTG	GTC	ATC	CTC	CTG	TCT	TAC	GCC	CGG	GTG	TCA	GTG	AAG	CTC			
	Leu	Leu	Pro	Leu	Leu	Val	Ile	Leu	Leu	Ser	Tyr	Ala	Arg	Val	Ser	Val	Lys	Leu			
	549			558			567			576			585			594					
	CGC	AAC	CGC	GTG	GTG	CCG	GGC	CGC	GTG	ACC	CAG	AGC	CAG	GCC	GAC	TGG	GAC	CGC			
	Arg	Asn	Arg	Val	Val	Pro	Gly	Arg	Val	Thr	Gln	Ser	Gln	Ala	Asp	Trp	Asp	Arg			
	603			612			621			630			639			648					
	GCT	CGG	CGC	CGG	CGC	ACC	TTC	TGC	TTC	CTG	GTG	GTG	GTC	GTG	GTG	GTG	TTC	ACC			
	Ala	Arg	Arg	Arg	Arg	Thr	Phe	Cys	Leu	Leu	Val	Val	Val	Val	Val	Val	Phe	Thr			
	657			666																	
	CTC	TGC	TGG	CTG	CCC	TTC	TTC	J'													
	Leu	Cys	Trp																		

7/53

[illegible]

Fig. 8



09576290-052300

Fig. 9

1	CATCGTCAAGCAGATGAAGATCATCCACGAGGATGGCTACTCCGAGGCCAGCAAAATT	60
1		1
61	CTGCCCCCTTCTTCCCGCGAGTGCTTTCCCGCTCTCCAAACCCCACTCCCAGGTGCCCATG	120
1	Met	1
121	GCCTCATCGACCACTCGGGGCCCCAGGGTTTCTGACTTATTTTCTGGGCTGCCGCGGCG	180
1	AlaSerSerThrThrArgGlyProArgValSerAspLeuPheSerGlyLeuProProAla	21
181	GTCACAACCTCCCGCCAACCAGAGCGCAGAGGCCTCGGCGGGCAACGGGTGGTGGCTGGC	240
21	ValThrThrProAlaAsnGlnSerAlaGluAlaSerAlaGlyAsnGlySerValAlaGly	41
241	GCGGACGCTCCAGCCGTCACGCCCTTCCAGAGCCTGCAGCTGGTGCATCAGCTGAAGGGG	300
41	AlaAspAlaProAlaValThrProPheGlnSerLeuGlnLeuValHisGlnLeuLysGly	61
301	CTGATCGTGCTGCTCTACAGCGTCGTGGTGGTTCGTGGGGCTGGTGGGCAACTGCCTGCTG	360
61	LeuIleValLeuLeuTyrSerValValValValValGlyLeuValGlyAsnCysLeuLeu	81
361	GTGCTGGTGATCGCGCGGGTGCGCCGGCTGCACAACGTGACGAACCTCCTCATCGGCAAC	420
81	ValLeuValIleAlaArgValArgArgLeuHisAsnValThrAsnPheLeuIleGlyAsn	101
421	CTGGCCTGTGCCGACGTGCTCATGTGCACCGCCTGCGTGCCGCTCAGCTGGCCATATGCC	480
101	LeuAlaLeuSerAspValLeuMetCysThrAlaCysValProLeuThrLeuAlaTyrAla	121
481	TTGAGCCACGCGGCTGGTGTTCGCGCGCGCCTGTGCCACCTGGTCTTCTTCCTGCAG	540
121	PheGluProArgGlyTrpValPheGlyGlyGlyLeuCysHisLeuValPhePheLeuGln	141
541	CCGGTCACCGTCTATGTGTGGGTGTTACGCTCACCACCATCGCAGTGGACCGCTACGTC	600
141	ProValThrValTyrValSerValPheThrLeuThrThrIleAlaValAspArgTyrVal	161
501	GTGCTGGTGACCCCGCTGAGGCGGCGCATCTCGCTGCGCCTCAGCGCCTACGCTGTGCTG	660
161	ValLeuValHisProLeuArgArgArgIleSerLeuArgLeuSerAlaTyrAlaValLeu	181
561	GCCATCTGGCGCTGTCCCGGTGCTGGCGCTGCCCGCCCGCTGCACACCTATCAGTG	720
181	AlaIleTrpAlaLeuSerAlaValLeuAlaLeuProAlaAlaValHisThrTyrHisVal	201
721	GAGCTCAAGCCGCACGACGTGCCCTCTGCGAGGAGTTCTGGGGCTCCCAGGAGCGCCAG	780
201	GluLeuLysProHisAspValArgLeuCysGluGluPheTrpGlySerGlnGluArgGln	221
781	CGCCAGCTCTACGCTGGGGGCTGCTGCTGGTCACTACCTGCTCCCTCTGCTGGTCACT	840
221	ArgGlnLeuTyrAlaTrpGlyLeuLeuLeuValThrTyrLeuLeuProLeuLeuValIle	241
841	CTCCTGTCTTACGTCCGGGTGTGAGTGAAGCTCCGCAACCGCTGGTGCCGGGCTGCGTG	900
241	LeuLeuSerTyrValArgValSerValLysLeuArgAsnArgValValProGlyCysVal	261
901	ACCAGAGCCAGGCCGACTGGGACCGCGCTCGGCGCGGCGCACCTTCTGCTGCTGGTG	960
261	ThrGlnSerGlnAlaAspTrpAspArgAlaArgArgArgThrPheCysLeuLeuVal	281
961	GTGGTCGTGGTGGTGTTCGCCGCTGCTGGCTGCCGCTGCACGCTCTTCAACCTGCTGCGG	1020
281	ValValValValValPheAlaValCysTrpLeuProLeuHisValPheAsnLeuLeuArg	301
1021	GACCTCGACCCCAAGCCATCGACCCCTACGCCTTTGGGCTGGTGCAGCTGCTCTGCCAC	1080
301	AspLeuAspProHisAlaIleAspProTyrAlaPheGlyLeuValGlnLeuLeuCysHis	321
1081	TGGCTCGCCATGAGTTCCGCCCTGCTACAACCCCTTCATCTACGCCTGGCTGCACGACAGC	1140
321	TrpLeuAlaMetSerSerAlaCysTyrAsnProPheIleTyrAlaTrpLeuHisAspSer	341
1141	TTCCGCGAGGAGCTGCGCAAACGTGGTTCGCTTGGCCCCGCAAGATAGCCCCCATGGC	1200
341	PheArgGluGluLeuArgLysLeuLeuValAlaTrpProArgLysIleAlaProHisGly	361
1201	CAGAATATGACCGTCAGCGTGGTCATCTGATGCCACTTAGCCAGGCCTTGGTCAAGGAGC	1260
361	GlnAsnMetThrValSerValValIle***	371
1261	TCCACTTCAACTGGCCTCTAGGGCACCCTCGAGGTCAATCTGGTGTCTATTCTCAGCA	1320
371		371
1321	CCAGAGCTAGC	1331
371		371



Fig. 10

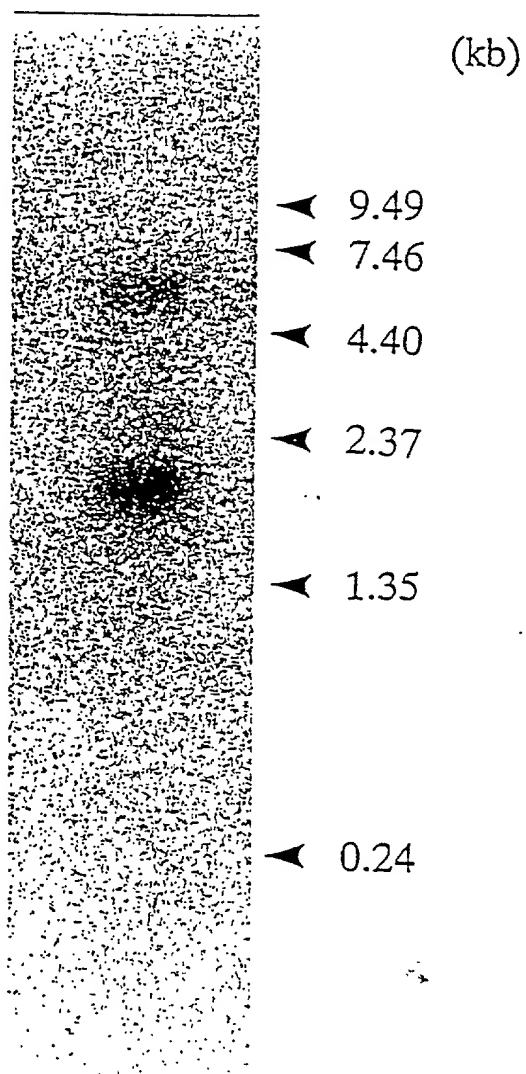
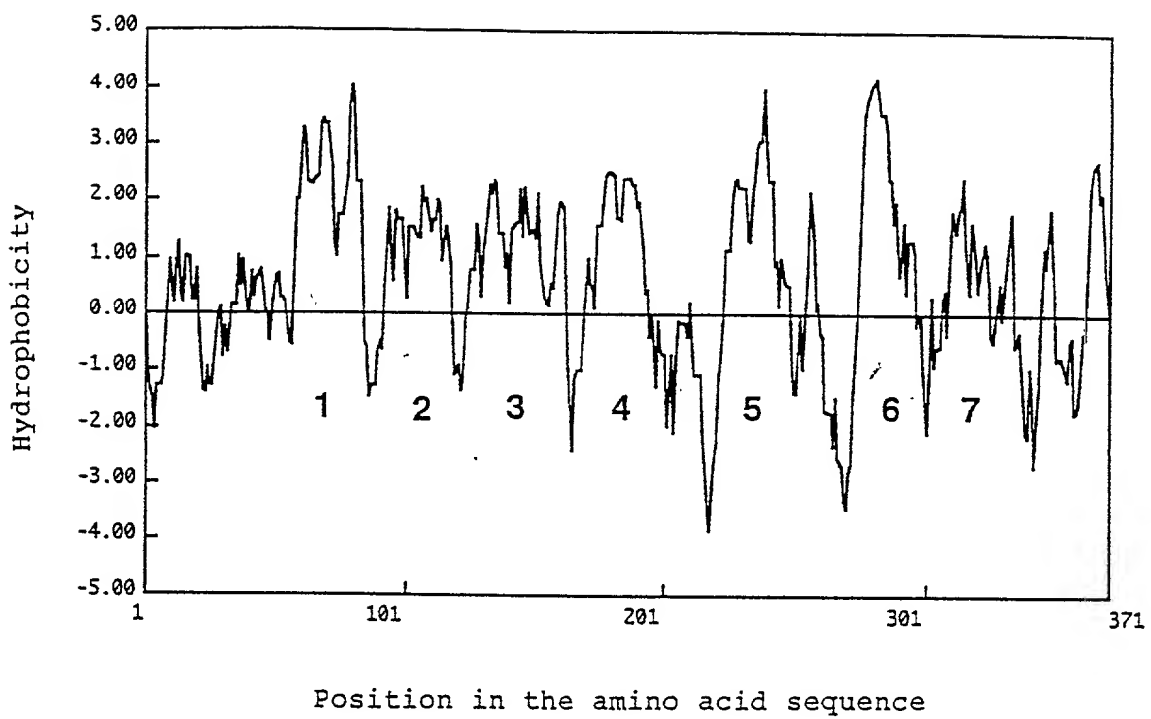


Fig. 11



CT 3'

	9				18				27				36				45				54			
5'	CTG	TGT	GTC	ATC	GCG	GTG	GAT	AGG	TAC	GTG	GTT	CTG	GTG	CAC	CCG	CTA	CGT	CGG						
	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---						
	Leu	Cys	Val	Ile	Ala	Val	Asp	Arg	Tyr	Val	Val	Leu	Val	His	Pro	Leu	Arg	Arg						
	63				72				81				90				99				108			
	CGC	ATT	TCA	CTG	AGG	CTC	AGC	GCC	TAC	GCG	GTG	CTG	GGC	ATC	TGG	GCT	CTA	TCT						
	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---						
	Arg	Ile	Ser	Leu	Arg	Leu	Ser	Ala	Tyr	Ala	Val	Leu	Gly	Ile	Trp	Ala	Leu	Ser						
	117				126				135				144				153				162			
	GCA	GTG	CTG	GCG	CTG	CCG	GCC	GCG	GTG	CAC	ACC	TAC	CAT	GTG	GAG	CTC	AAG	CCC						
	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---						
	Ala	Val	Leu	Ala	Leu	Pro	Ala	Ala	Val	His	Thr	Tyr	His	Val	Glu	Leu	Lys	Pro						
	171				180				189				198				207				216			
	CAC	GAC	GTG	AGC	CTC	TGC	GAG	GAG	TTC	TGG	GGC	TCG	CAG	GAG	CGC	CAA	CGC	CAG						
	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---						
	His	Asp	Val	Ser	Leu	Cys	Glu	Glu	Phe	Trp	Gly	Ser	Gln	Glu	Arg	Gln	Arg	Gln						
	225				234				243				252				261				270			
	ATC	TAC	GCC	TGG	GGG	CTG	CTT	CTG	GGC	ACC	TAT	TTG	CTC	CCC	CTG	CTG	GCC	ATC						
	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---						
	Ile	Tyr	Ala	Trp	Gly	Leu	Leu	Leu	Gly	Thr	Tyr	Leu	Leu	Pro	Leu	Leu	Ala	Ile						
	279				288				297				306				315				324			
	CTC	CTG	TCT	TAC	GTA	CGG	GTG	TCA	GTG	AAG	CTG	AGG	AAC	CGC	GTG	GTG	CCT	GGC						
	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---						
	Leu	Leu	Ser	Tyr	Val	Arg	Val	Ser	Val	Lys	Leu	Arg	Asn	Arg	Val	Val	Pro	Gly						
	333				342				351				360				369				378			
	AGC	GTG	ACC	CAG	AGT	CAA	GCT	GAC	TGG	GAC	CGA	GCG	CGT	CGC	CGC	CGC	ACT	TTC						
	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---						
	Ser	Val	Thr	Gln	Ser	Gln	Ala	Asp	Trp	Asp	Arg	Ala	Arg	Arg	Arg	Arg	Thr	Phe						
	387				396				405				414				423				432			
	TGT	CTG	CTG	GTG	GTG	GTG	GTA	GTG	TTC	ACG	CTC	TGC	TGG	CTG	CCC	TTC	TAC							
	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---							
	Cys	Leu	Leu	Val	Val	Val	Val	Val	Phe	Thr	Leu	Cys	Trp	Leu	Pro	Phe	Tyr							

Fig. 13

p19P2 pg3-2/pg1-10 p5S38	1 1 -79	<b>VGMVGNVLTIV</b> <b>VGMVGNVLTIV</b> .....	10 20 .....	<b>LVITARVRRLI</b> <b>LVITARVRRLY</b> .....	30 40 .....	<b>INVTNFIIGNL</b> <b>INVTNFIIGNL</b> .....	50 50 50 -30	
p19P2 pg3-2/pg1-10 p5S38	51 51 -29	<b>EPRGWNVEGGG</b> <b>EPRGWNVEGGG</b> .....	60 70 .....	<b>LCHLVFFLQ</b> <b>LCHLVFFLQ</b> .....	80 90 .....	<b>VTYVSVFTL</b> <b>VTYVSVFTL</b> .....	100 100 100 21	
p19P2 pg3-2/pg1-10 p5S38	101 101 22	<b>LRLSAYAVLA</b> <b>LRLSAYAVLA</b> <b>LRLSAYAVLS</b>	110 120 .....	<b>IMVLSAVLAL</b> <b>IMVLSAVLAL</b> <b>IMVLSAVLAL</b>	130 140 .....	<b>PAVHTYHVE</b> <b>PAVHTYHVE</b> <b>PAVHTYHVE</b>	150 150 71	
p19P2 pg3-2/pg1-10 p5S38	151 151 72	<b>GLLV</b> <b>QIYAMGLLV</b> <b>QIYAMGLLV</b>	160 170 .....	<b>TYLLPLLVTL</b> <b>TYLLPLLVTL</b> <b>TYLLPLLVTL</b>	180 190 .....	<b>LSYVRVSVKL</b> <b>LSYVRVSVKL</b> <b>LSYVRVSVKL</b>	200 200 121	
p19P2 pg3-2/pg1-10 p5S38	201 201 122	<b>RRRTTECLLV</b> <b>RRRTTECLLV</b> <b>RRRTTECLLV</b>	210 220 .....	<b>VVVVFALCWL</b> <b>VVVVFALCWL</b> <b>VVVVFALCWL</b>	230 240 .....	<b>PY</b> <b>PY</b> <b>PY</b>	250 250 171	

Fig. 14

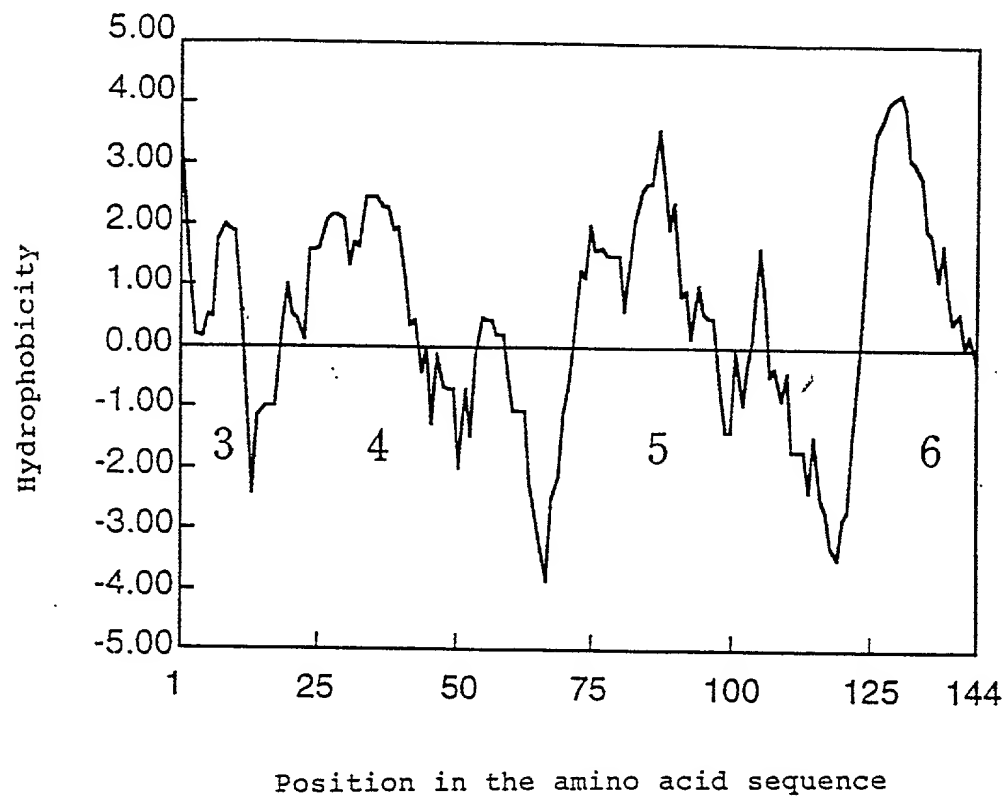
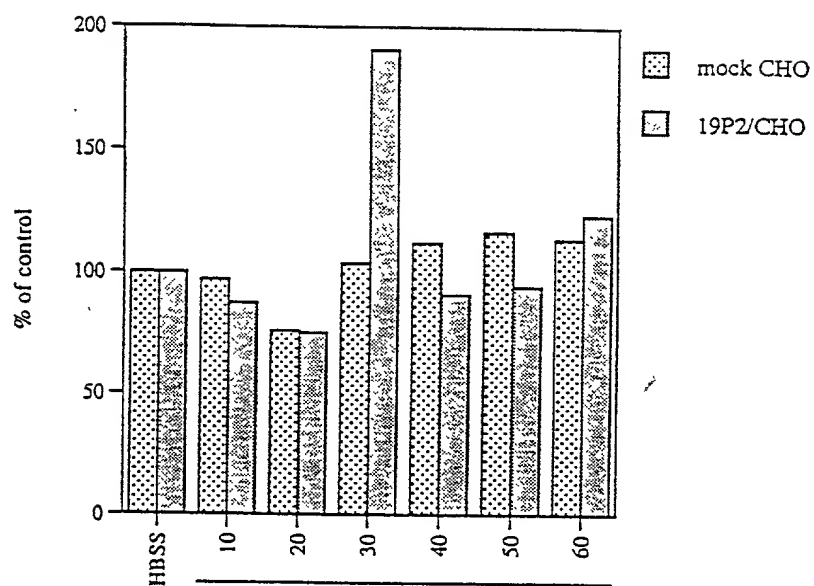




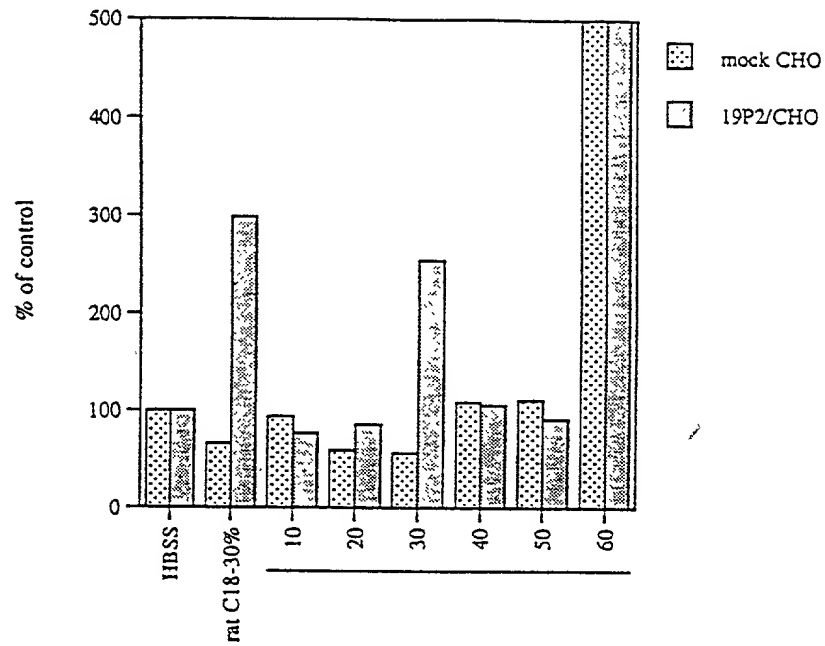
Fig. 16



rat whole brain extract

C<sub>18</sub>-column CH<sub>3</sub>CN elution (%)

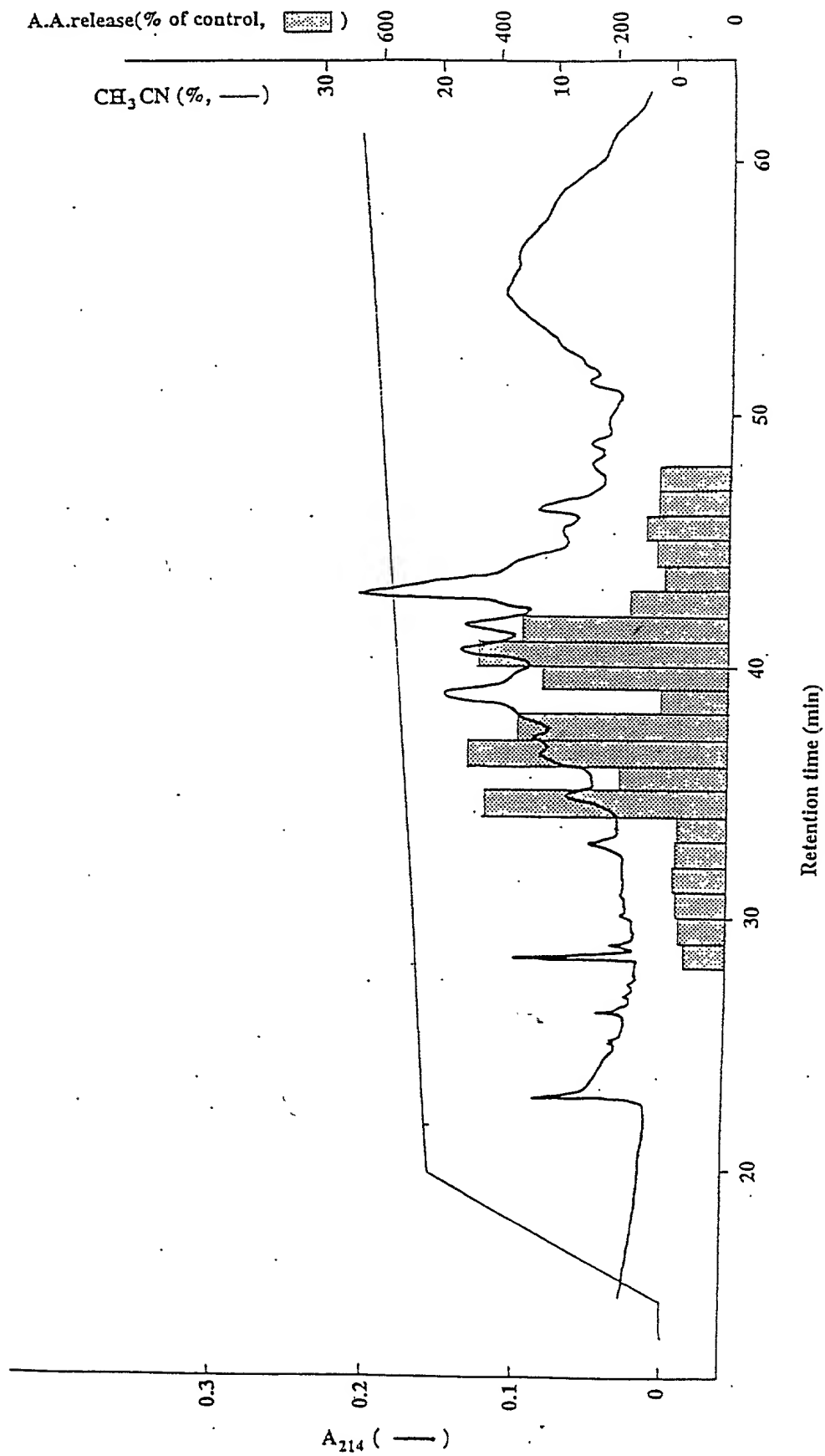
Fig. 17



bovine hypothalamus extract  
C<sub>18</sub>-column CH<sub>3</sub>CN elution (%)



Fig. 18



19/53

Fig. 19

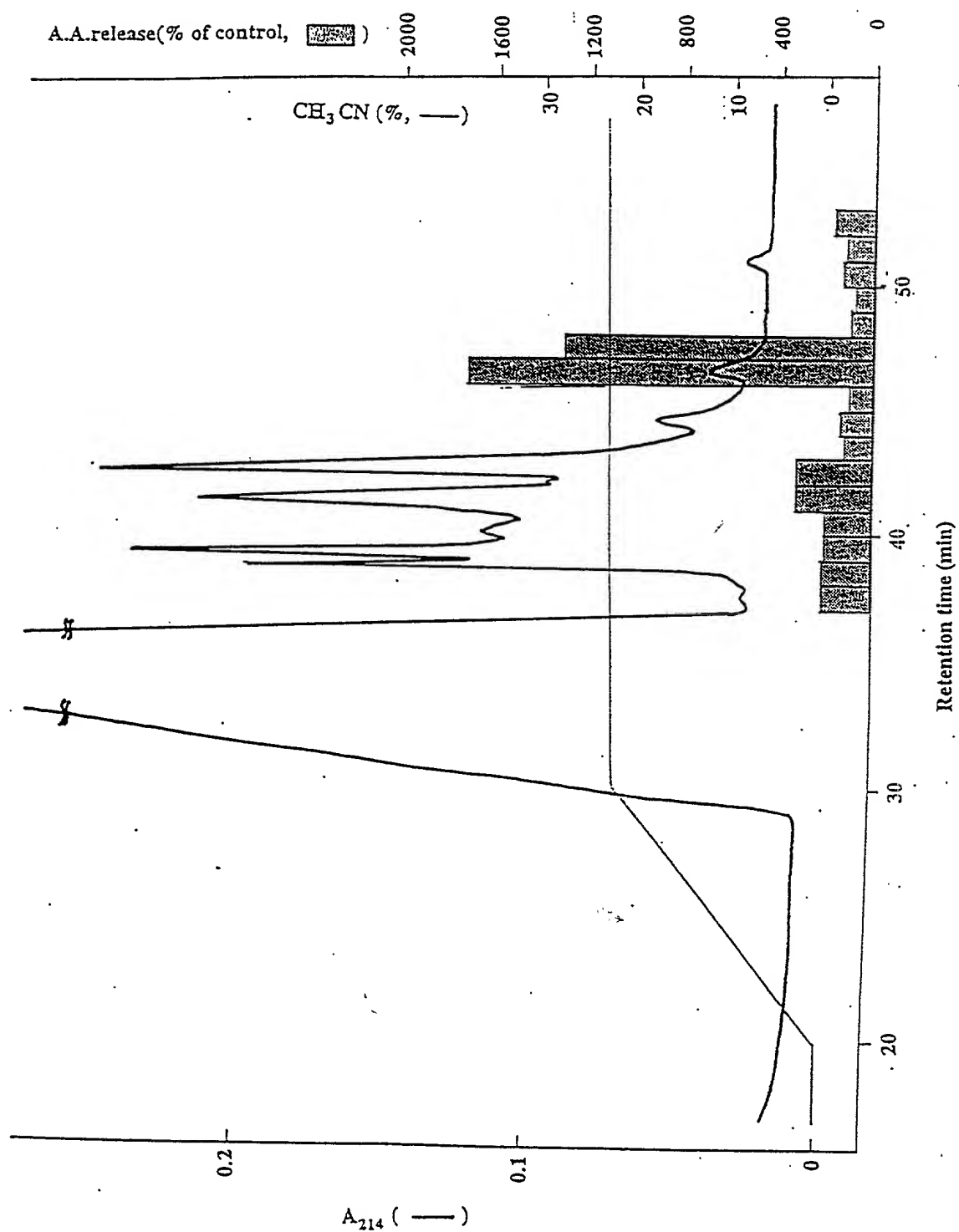


Fig. 20

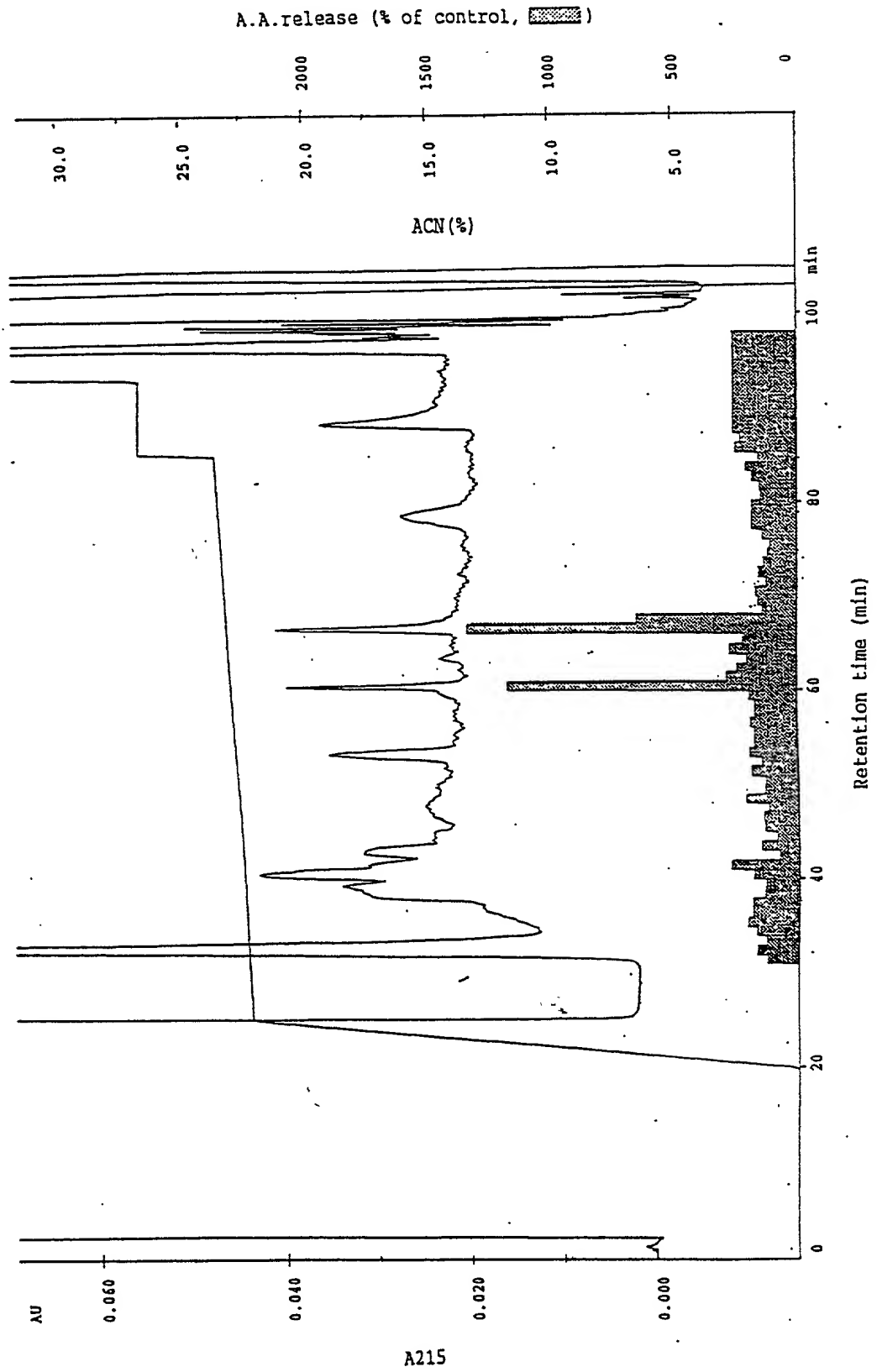


Fig. 21

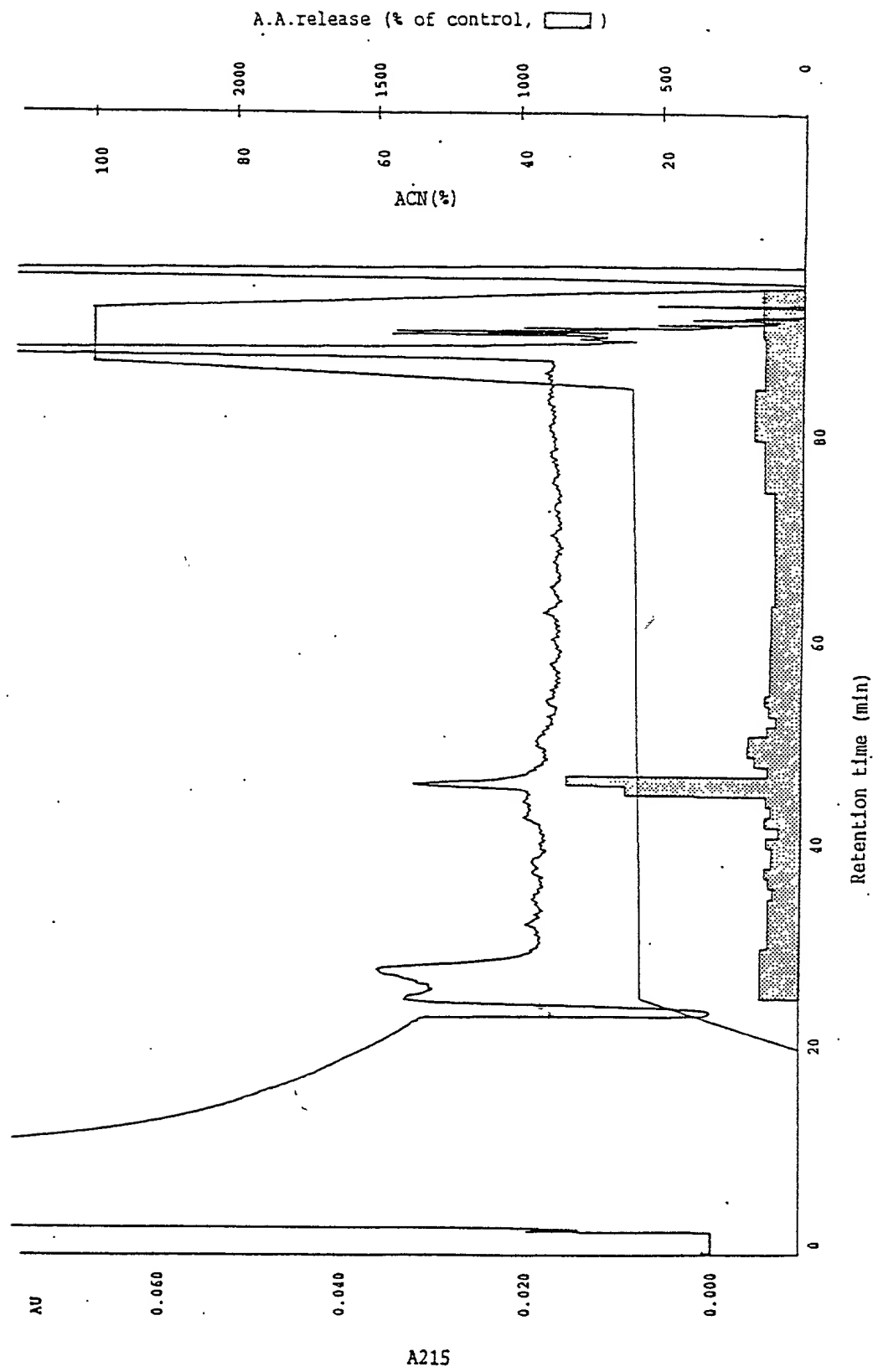


Fig. 22

```


P5-1
      9           18           27           36           45           54
GCC CAC CAG CAC TCC ATG GAG ATC CGC ACC CCC GAC ATC AAC CCT GCC TGG TAC
----
Ala His Gln His Ser Met Glu Ile Arg Thr Pro Asp Ile Asn Pro Ala Trp Tyr
      63           72
GCG GGC CGT GGG ATC CGG CCC G 3'
----
Ala Gly Arg Gly Ile Arg Pro
P3-2

```

Fig. 23

```

1  GTGGAATGAAGGCGGTGGGGGCCTGGCTCCTCTGCCTGCTGCTGCTGGGGCCTGGCCCTG      59
1      MetLysAlaValGlyAlaTrpLeuLeuCysLeuLeuLeuLeuGlyLeuAlaLeu      18

60  CAGGGGGCTGCCAGCAGAGCCCACCAGCACTCCATGGAGATCCGCACCCCCGACATCAAC      119
19  GlnGlyAlaAlaSerArgAlaHisGlnHisSerMetGluIleArgThrProAspIleAsn      38
                                     
                                     PDN

120 CCTGCCT      126
39  ProAla      40

```

Fig. 24(a)

1 GTGGAATGAAGCGGTGGGGCCTGGCTCCTCTGCCTGCTGCTGCTGGGCCTGGCCCTG 59  
 1 MetLysAlaValGlyAlaTrpLeuLeuCysLeuLeuLeuLeuGlyLeuAlaLeu 18  
 60 CAGGGGGCTGCCAGCAGAGCCCACCAGCACTCCATGGAGATCCGCACCCCCGACATCAAC 119  
 19 GlnGlyAlaAlaSerArgAlaHisGlnHisSerMetGluIleArgThrProAspIleAsn 38  
 120 CCTGCCTGGTACGCRGGCCCGTGGGATCCGGCCCGTGGGCTCGCTTCGGCCGGCGAAGAGCT 179  
 39 ProAlaTrpTyrAlaGlyArgGlyIleArgProValGlyArgPheGlyArgArgAla 58  
 180 GCCCCGGGGGACGGACCCAGGCCTGGCCCCCGCGTGTGCCGGCCTGCTTCCGCCTGGAA 239  
 59 AlaProGlyAspGlyProArgProGlyProArgArgValProAlaCysPheArgLeuGlu 78  
 240 GGCGGYGCTGAGCCCTCCCGAGCCCTCCCGGGGCGGCTGACGGCCCAGCTGGTCCAGGAA 299  
 79 GlyGlyAlaGluProSerArgAlaLeuProGlyArgLeuThrAlaGlnLeuValGlnGlu 98  
 300 TAACAGCGGGAGCCTGCCCCCACCCTCCTCCTCCACCAGCCACCTTCCCTCCAGTCCT 359  
 98 98  
 360 AATAAAAGCAGCTGGCTTGTT 380  
 98 98

002250"0524660

Fig. 24(b)

1	GTGGAATGAAGGCGGTGGGGGGCCTGGCTCCTCTGCCTGCTGCTGGGCGCTGGCCCTG	59
1	MetLysAlaValGlyAlaTrpLeuLeuCysLeuLeuLeuLeuGlyLeuAlaLeu	18
60	CAGGGGGCTGCCAGCAGAGCCCACCAGCACTCCATGGAGATCCGCACCCCCGACATCAAC	119
19	GlnGlyAlaAlaSerArgAlaHisGlnHisSerMetGluIleArgThrProAspIleAsn	38
120	CCTGCCTGGTACGCRGGCCGTGGGATCCGGCCCCGTGGCGCTTCGGCCGCGGAAGAGCT	179
39	ProAlaTrpTyrAlaGlyArgGlyIleArgProValGlyArgPheGlyArgArgArgAla	58
180	GCCCTGGGGGACGGACCCAGGCCTGGCCCCCGCGTGTGCCGGCCTGCTTCCGCCTGGAA	239
59	AlaLeuGlyAspGlyProArgProGlyProArgArgValProAlaCysPheArgLeuGlu	78
240	GGCGGYGCTGAGCCCTCCCGAGCCCTCCCGGGGCGGCTGACGGCCAGCTGGTCCAGGAA	299
79	GlyGlyAlaGluProSerArgAlaLeuProGlyArgLeuThrAlaGlnLeuValGlnGlu	98
300	TAACAGCGGGAGCCTGCCCCCCACCCCTCCTCCTCCACCAGCCACCTTCCCTCCAGTCCT	359
98		98
360	AATAAAAGCAGCTGGCTTGTT	380
98		98



Fig. 25

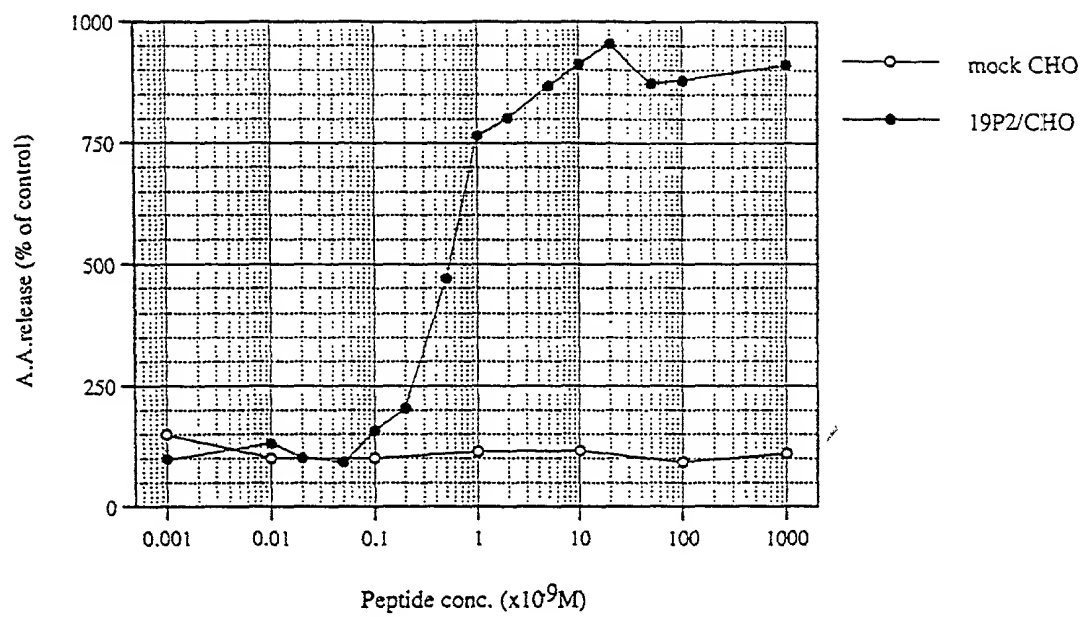


Fig. 26

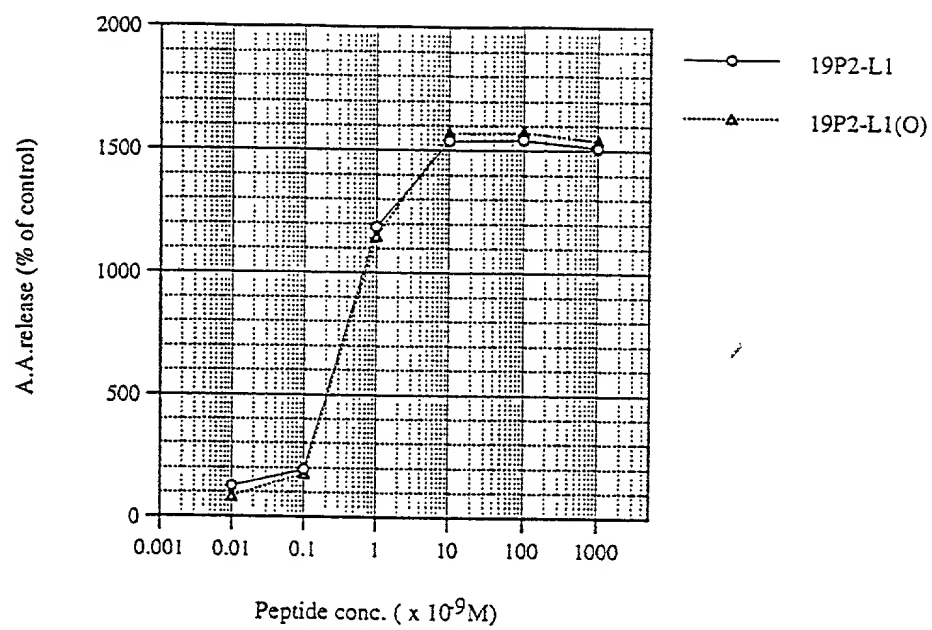


Fig. 27

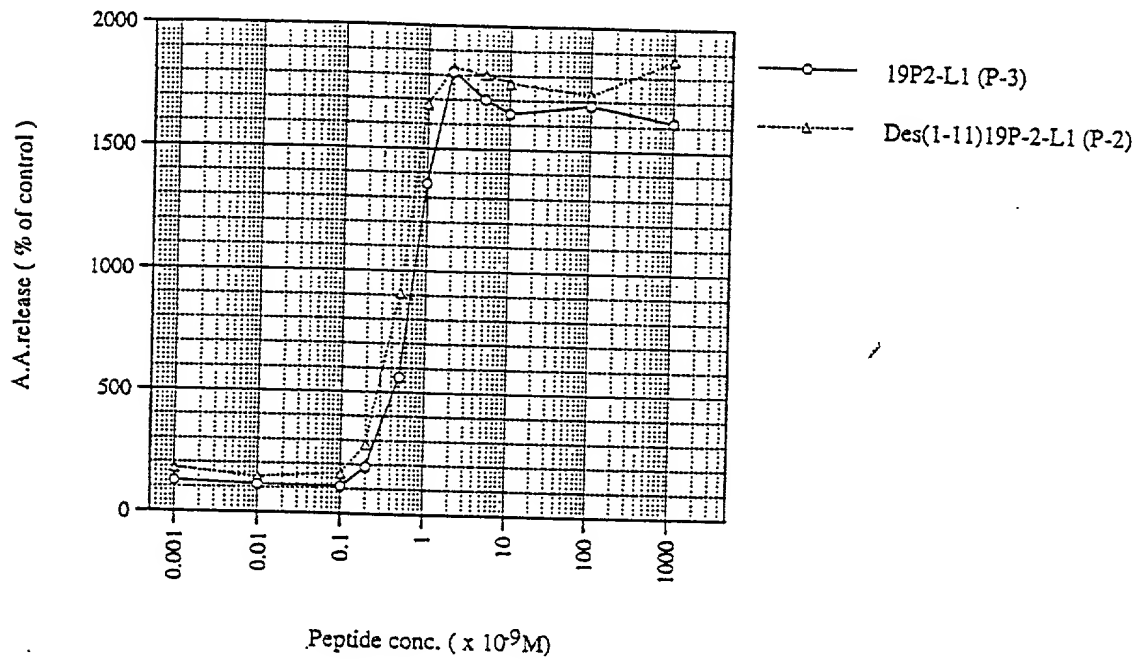


Fig. 28

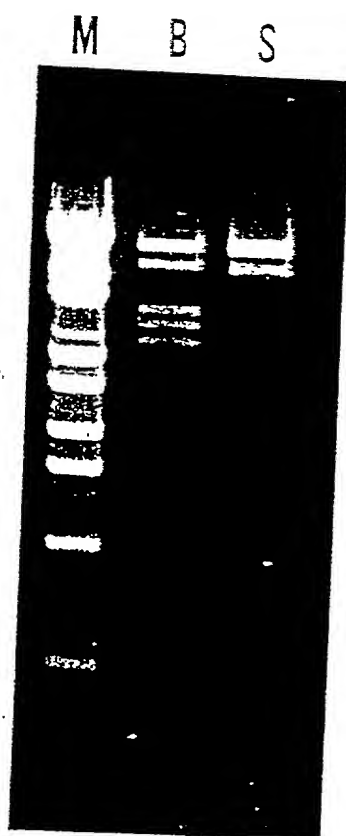


Fig. 29

10	20	30	40	50	60
ATGAAGGCGG	TGGGGGCCTG	GCTCCTCTGC	CTGCTGCTGC	TGGGCCTGGC	CCTGCAGGGG
70	80	90	100	110	120
GCTGCCAGCA	GAGCCCACCA	GCACTCCATG	GAGATCCGCA	GTGAGTGTCT	AGCCCCGCCC
130	140	150	160	170	180
CTGCCCCCAG	GGGTCACAGG	GGGGGCCTGG	CCACTTCCTG	GGCTGGGACA	TCCTTGCTAA
190	200	210	220	230	240
GCATCCTGGG	GTTGGGGTTT	GGCCTCCTGT	TCCCCAGACC	CTTCCCCCAG	GTGGCCCCGA
250	260	270	280	290	300
CAGGTGCTCC	CAAGGGTCCC	GGCCCAGCAC	ACGGGGGAGG	GTCACTCCTC	ACCACACGGG
310	320	330	340	350	360
TGGCCTGGGG	CTGAGTGAC	GTCACCCATG	AGAACGGGGC	TGTGAGGACA	GGAAAGGAAG
370	380	390	400	410	420
GGGAGTGTGT	CCTGGTGTGA	GTCCTGAAATC	CTACTTCCCA	AAGCCACCCC	AGCACCAGAA
430	440	450	460	470	480
ATGGGCGCTC	CGGGTGAACC	TCCTGTGCGG	GTGGGTGGTC	CTGGCATGGC	CTGGGGCACA
490	500	510	520	530	540
GGCAGCCATG	AGCTGAGCAC	ACACCCGGCC	CGGCCACCAG	GGCTGTATGC	TCCAGGGCAC
550	560	570	580	590	600
AGGCCTCCAT	GCGCTCTTCT	CTCTCTTTCC	AGCCCCCGAC	ATCAACCCTG	CCTGGTACGC
610	620	630	640	650	660
AGGCCGTGGG	ATCCGGCCCCG	TGGGCGCCTT	CGGCCGGCGA	AGAGCTGCCC	TGGGGGACGG
670	680	690	700	710	720
ACCCAGGCCT	GGCCCCCGGC	GTGTGCCGGC	CTGCTTCCGC	CTGGAAGGCG	GTGCTGAGCC
730	740	750	760	770	780
CTCCCGAGCC	CTCCCGGGGC	GGCTGACGGC	CCAGCTGGTC	CAGGAATAA..	.....

Fig. 30

genome cDNA	1	10	20	30	40	50	50
		ATGAAGGCGG	TGGGGGCGCTG	GCTCCTCTGC	CTGCTGCTGC	TGGGCCTGGC	
genome cDNA	1	60	70	80	90	100	100
		CCTGCAGGGG	GCTGCCAGCA	GAGCCACCA	GCACTCCATG	GAGATCCGCA	
genome cDNA	51	110	120	130	140	150	150
		GTGAGTGTCT	AGCCCCGCCC	CTGCCCCCAG	GGGTACACAGG	GGGGCCCTGG	
genome cDNA	101	160	170	180	190	200	200
		CCACTTCCTG	GGCTGGGACA	TCCTTGCTAA	GCATCCTGGG	GTTGGGGTTT	
genome cDNA	201	210	220	230	240	250	250
		GGCCTCCTGT	TCCCCAGACC	CTTCCCCCAG	GTGGCCCGGA	CAGGTGCTCC	
genome cDNA	251	260	270	280	290	300	300
		CAAGGTCCCC	GGCCCAGCAC	ACGGGGGAGG	GTCCTCCTC	ACCACACGGG	
genome cDNA	301	310	320	330	340	350	350
		TGGCCTGGGG	CTGAGTGCAC	GTCACCCATG	AGAACGGGGC	TGTGAGGACA	
genome cDNA	351	360	370	380	390	400	400
		GGAAAGGAAG	GGGAGTGTGT	CCTGGTGTGA	GTCTGAAATC	CTACTTCCCA	
genome cDNA	401	410	420	430	440	450	450
		AAGCCACCCC	AGCACCAGAA	ATGGGCGCTC	CGGGTGAACC	TCCTGTGCGG	
genome cDNA	451	460	470	480	490	500	500
		GTGGGTGGTC	CTGGCATGGC	CTGGGCGACA	GGCAGCCATG	AGCTGAGCAC	
genome cDNA	501	510	520	530	540	550	550
		ACACCCGGCC	CGGCCACCAG	GGCTGTATGC	TCCAGGGCAC	AGGCCTCCAT	
genome cDNA	551	560	570	580	590	600	600
		GCGCTCTTCT	CTCTCTTTCC	AGCCCCCGAC	ATCAACCCCTG	CCTGGTACGC	
genome cDNA	601	610	620	630	640	650	650
		AGGCCGTGGG	ATCCGGCCCCG	TGGGCGGCTT	CGGCCGGCGA	AGAGCTGCC	
genome cDNA	651	660	670	680	690	700	700
		TGGGGGACGG	ACCCAGGCCCT	GGCCCCCGGC	GTGTGCCGGC	CTGCTTCCGC	
genome cDNA	701	710	720	730	740	750	750
		CTGGAAGGCG	GTGCTGAGCC	CTCCCGAGCC	CTCCCGGGGC	GGCTGACGGC	
genome cDNA	751	760	770	780	790	800	800
		CCAGCTGGTC	CAGGAATAA.	.....	.....	.....	

5'	9				18				27				36				45				54			
	ATG	AAG	GCG	GTG	GGG	GCC	TGG	CTC	CTC	TGC	CTG	CTG	CTG	CTG	GGC	CTG	GCC	CTG						
	M	K	A	V	G	A	W	L	L	C	L	L	L	L	G	L	A	L						
	63				72				81				90				99				108			
	CAG	GGG	GCT	GCC	AGC	AGA	GCC	CAC	CAG	CAC	TCC	ATG	GAG	ATC	CGC	ACC	CCC	GAC						
	Q	G	A	A	S	R	A	H	Q	H	S	M	E	I	R	T	P	D						
	117				126				135				144				153				162			
	ATC	AAC	CCT	GCC	TGG	TAC	GCA	GGC	CGT	GGG	ATC	CGG	CCC	GTG	GGC	CGC	TTC	GGC						
	I	N	P	A	W	Y	A	G	R	G	I	R	P	V	G	R	F	G						
	171				180				189				198				207				216			
	CGG	CGA	AGA	GCT	GCC	CTG	GGG	GAC	GGA	CCC	AGG	CCT	GGC	CCC	CGG	CGT	GTG	CCG						
	R	R	R	A	A	L	G	D	G	P	R	P	G	P	R	R	V	P						
	225				234				243				252				261				270			
	GCC	TGC	TTC	CGC	CTG	GAA	GGC	GGT	GCT	GAG	CCC	TCC	CGA	GCC	CTC	CCG	GGG	CGG						
	A	C	F	R	L	E	G	G	A	E	P	S	R	A	L	P	G	R						
	279				288				297															
	CTG	ACG	GCC	CAG	CTG	GTC	CAG	GAA	TAA	3'														
	L	T	A	Q	L	V	Q	E	*															

Fig. 32

1	GGCATCATCCAGGAAGACGGAGCATGGCCCTGAAGACGTGGCTTCTGTGCTTGCTGCTG	59
1	MetAlaLeuLysThrTrpLeuLeuCysLeuLeuLeu	12
60	CTAAGCTTGGTCCTCCCAGGGGCTTCCAGCCGAGCCCACCAGCACTCCATGGAGACAAGA	119
13	LeuSerLeuValLeuProGlyAlaSerSerArgAlaHisGlnHisSerMetGluThrArg	32
120	ACCCCTGATATCAATCCTGCCTGGTACACGGGCGCGGGATCAGGCCTGTGGGCGCTTC	179
33	ThrProAspIleAsnProAlaTrpTyrThrGlyArgGlyIleArgProValGlyArgPhe	52
180	GGCAGGAGAAGGGCAACCCCGAGGGATGTCACTGGACTTGGCCAACTCAGCTGCCTCCCA	239
53	GlyArgArgArgAlaThrProArgAspValThrGlyLeuGlyGlnLeuSerCysLeuPro	72
240	CTGGATGGACGCACCAAGTTCTCTCAGCGTGGATAACACCCAGCTCGAGAAGACAGTGC	299
73	LeuAspGlyArgThrLysPheSerGlnArgGly***	83
300	TGCTGAGCCCAAGCCACACTCCCTGTCCCCTGCAGACCCTCCTCTACCCCTCCCTCTCCT	359
83		83
360	CTGCT	364
83		83



Fig. 33

bovine.aa		M K A V G A W L L					
		10	20	30	40	50	
bovine.seq	-18	.....GT	GGAATGAAGG	CGGTGGGGGC	CTGGCTCCTC		32
rat.seq	1	GGCATCATCC	AGGAAGACGG	AGCATG---G	CCCTGAAGAC	<u>GTGGCTCTCG</u>	50
bovine.aa		C L L L L G L A L Q G A A S R A H					
		60	70	80	90	100	
bovine.seq	33	TGCTGCTGCT	TGCTGGGCT	GGCCCTGCAG	GGGGCTGCCA	GCAGAGCCCA	82
rat.seq	51	<u>TGCTTGTCTG</u>	<u>TGCTAAGCTT</u>	GGTCTCTCCA	GGGGCTTCCA	GCCGAGCCCA	100
		R1					
bovine.aa		Q H S M E I R T P D I N P A W Y A					
		110	120	130	140	150	
bovine.seq	83	CCAGCACTCC	ATGGAGATCC	GCACCCCGA	CATCAACCCT	GCCTGGTACG	132
rat.seq	101	CCAGCACTCC	ATGGAGACAA	GAACCCCTGA	TATCAATCCT	<u>GCCTGGTACA</u>	150
		R3					
bovine.aa		G R G I R P V G R F G R R R A A					
		160	170	180	190	200	
bovine.seq	133	CGGGCCGTGG	GATCCGGGCC	GTGGGCCGCT	TGGGCCGGCG	AAGAGCTGCC	182
rat.seq	151	<u>CGGGCCGCGG</u>	<u>GATCAGGCCT</u>	<u>GTGGGCCGCT</u>	<u>TGGGCAGGAG</u>	<u>AAGGGCAACC</u>	200
		R4					
bovine.aa		P G D G P R P G P R R V P A C F R					
		210	220	230	240	250	
bovine.seq	183	CCGGGGGACG	GACCCAGGCC	TGGCCCCCGG	CGTGTGCCCG	CCTGCTTCCG	232
rat.seq	201	CCGAGGGATG	TCACTGGACT	TGGC-----	---CAACTCA	GCTGCCTCCC	250
bovine.aa		L E G G A E P S R A L P G R L T A					
		260	270	280	290	300	
bovine.seq	233	CCTGGAAGGC	GGCGCTGAGC	CCTCCCGAGC	CCTCCCGGGG	CGGCTGACGG	282
rat.seq	251	ACTGGATGGA	CGCACCAAGT	TCTCTCAGCG	TGGATAACAC	CCCAGCTCGA	300
bovine.aa		Q L V Q E *					
		310	320	330	340	350	
bovine.seq	283	CCAGCTGGT	CCAGGAATAA	CAGCGGGAGC	CTGCCCCCCA	CCGCTCTCTC	332
rat.seq	301	GAAGACAGTG	CTGCTGAGCC	CAAGCCCACA	CTCCCTGTCC	CCTGCAGACC	350
		360	370	380	390	400	
bovine.seq	333	TCCACCAGCC	ACCTTCCCTC	CAGTCTTAAT	AAAAGCAGCT	GGCTTGT..	382
rat.seq	351	CTCCTCTACC	CTCCCTCTCC	TCTGT....	.....	.....	400

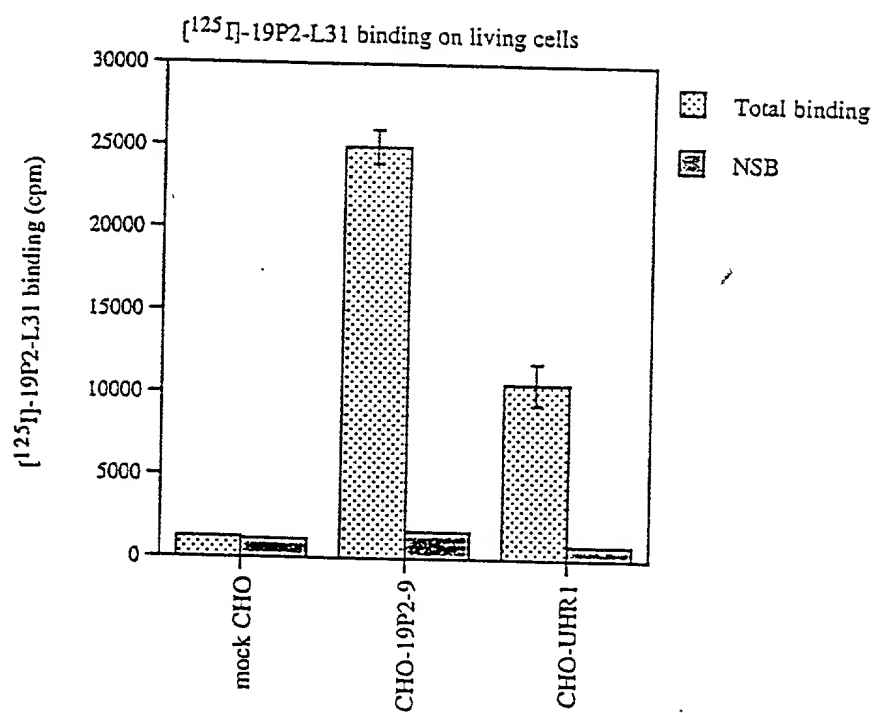
Fig. 34

1	GGCCTCCTCGGAGGAGCCAAGGGATGAAGGTGCTGAGGGGCTGGCTCCTGTGCCTGCTG	59
1	MetLysValLeuArgAlaTrpLeuLeuCysLeuLeu	12
60	ATGCTGGGCCTGGCCCTGCGGGGAGCTGCAAGTCGTACCCATCGGCACCTCCATGGAGATC	119
13	MetLeuGlyLeuAlaLeuArgGlyAlaAlaSerArgThrHisArgHisSerMetGluIle	32
120	CGCACCCCTGACATCAATCCTGCCTGGTACGCCAGTCGCGGGATCAGGCCTGTGGGCCCCG	179
33	ArgThrProAspIleAsnProAlaTrpTyrAlaSerArgGlyIleArgProValGlyArg	52
180	TTCGGTCGGAGGAGGGCAACCCTGGGGGACGTCCCCAAGCCTGGCCTGCACCCCGGCTG	239
53	PheGlyArgArgArgAlaThrLeuGlyAspValProLysProGlyLeuArgProArgLeu	72
240	ACCTGCTTCCCCCTGGAAGGCGGTGCTATGTCTGTCCCAGGATGGCTGACAGCCAGCTTGT	299
73	ThrCysPheProLeuGluGlyGlyAlaMetSerSerGlnAspGly***	87
300	CAAGAACTCACTCTGGAGCCTCCCCACCCACCCCTCTCCTCTCCTTCGGGCTCCTTTC	359
87		87
360	CC	361
87		87

Fig. 35

		10	20	30	40	50	
bovine.aa	1	MKAVGAWLLC	LLLGLALQG	AASRAHQHSM	EIRTPDINPA	WYAGRGIRFV	50
rat.aa	1	M-ALKTWLLC	LLLLLVLPFG	ASSRAHQHSM	ETRTPDINPA	WYTGGRGIRFV	50
human.aa	1	MKVLRAWLLC	LLMLGLALRG	AASRTHRHSM	EIRTPDINPA	WYASRGIRFV	50
		60	70	80	90	100	
bovine.aa	51	GRFGRRRAAP	GDGPRPGFRR	VPACFRLEGG	AEPSRALPGR	LTAQLVQE*	100
rat.aa	51	GRFGRRRAAP	RDVTGLG---	QLSCLPLDGR	TKFSQSG*..	.....	100
human.aa	51	GRFGRRRAATL	GDVFKPGLRP	RLTCFPLEGG	AMSSQDG*..	.....	100

Fig. 36



cells;  $0.5 \times 10^7$  cells/ml

$[^{125}\text{I}]\text{-19P2-L31}$ ; 200pM (avg. 63857.3cpm)

NSB; 200nM ( $\times 1,000$ )

reaction; RT, 2.5hr

in HBSS + 0.05% BSA + 0.05% CHAPS

in 100  $\mu\text{l}$

Fig. 37

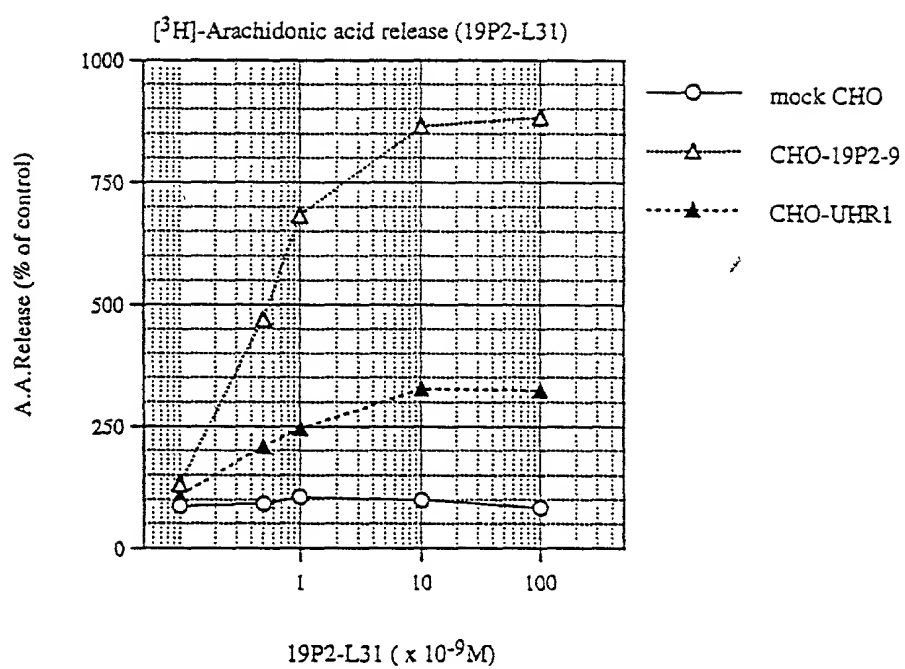


Fig. 38

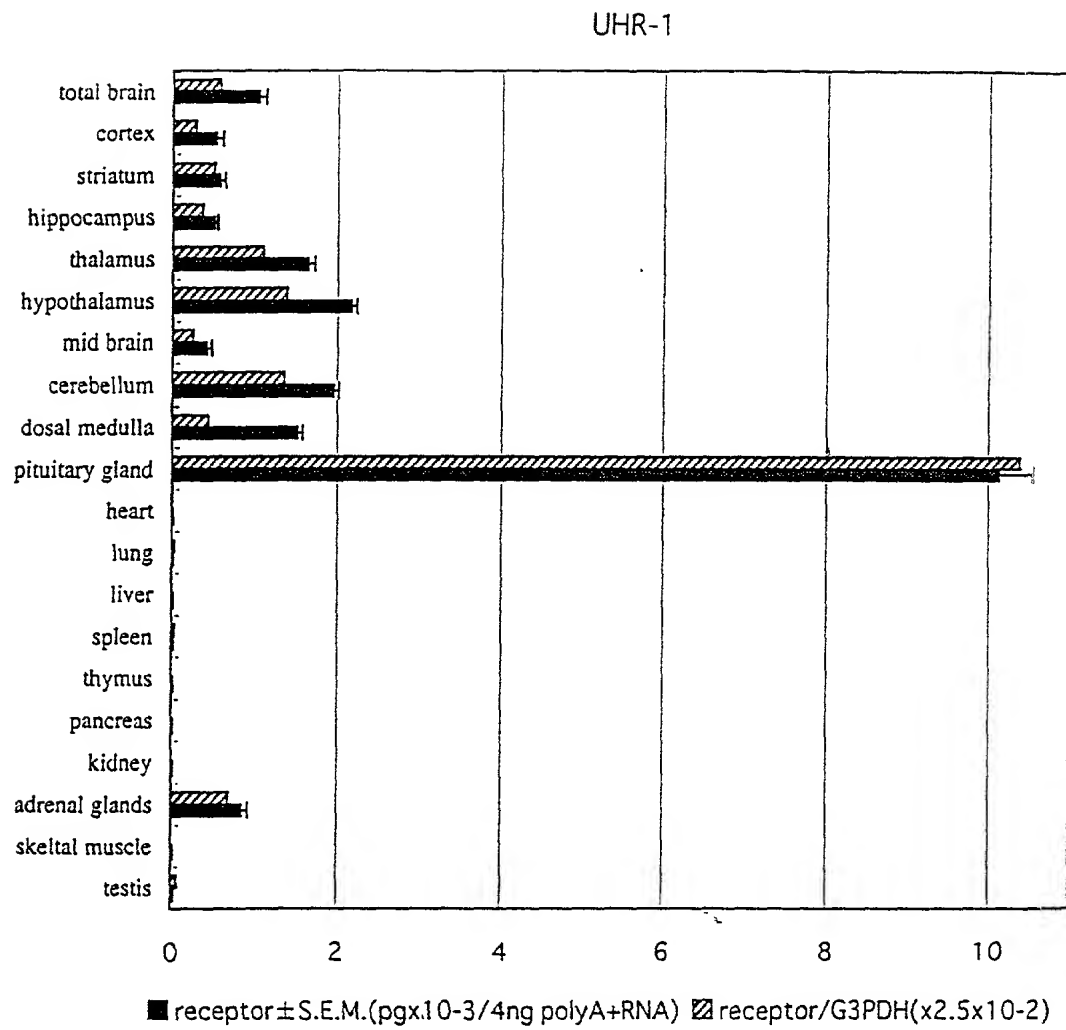


Fig. 39

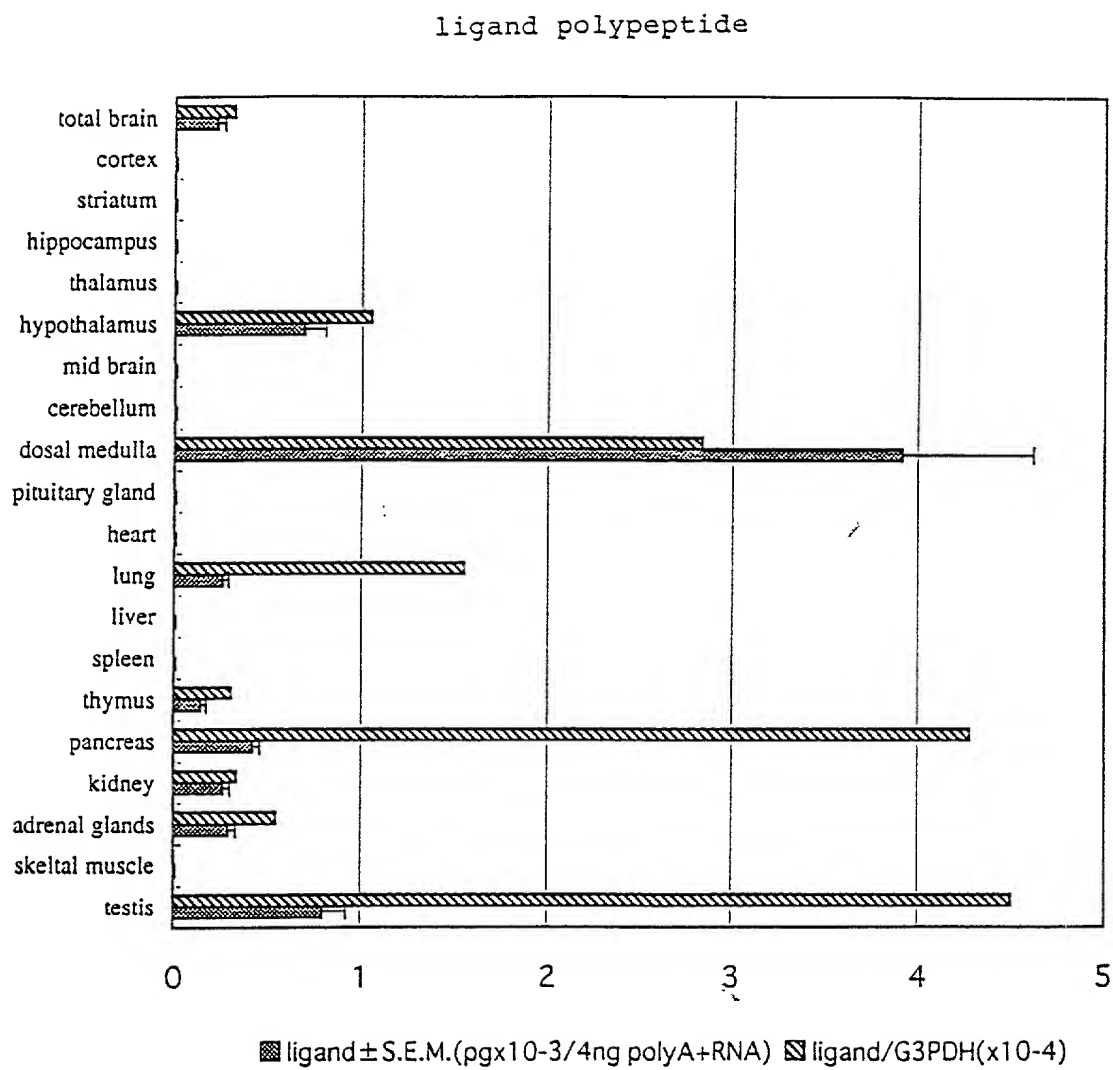


Fig. 40

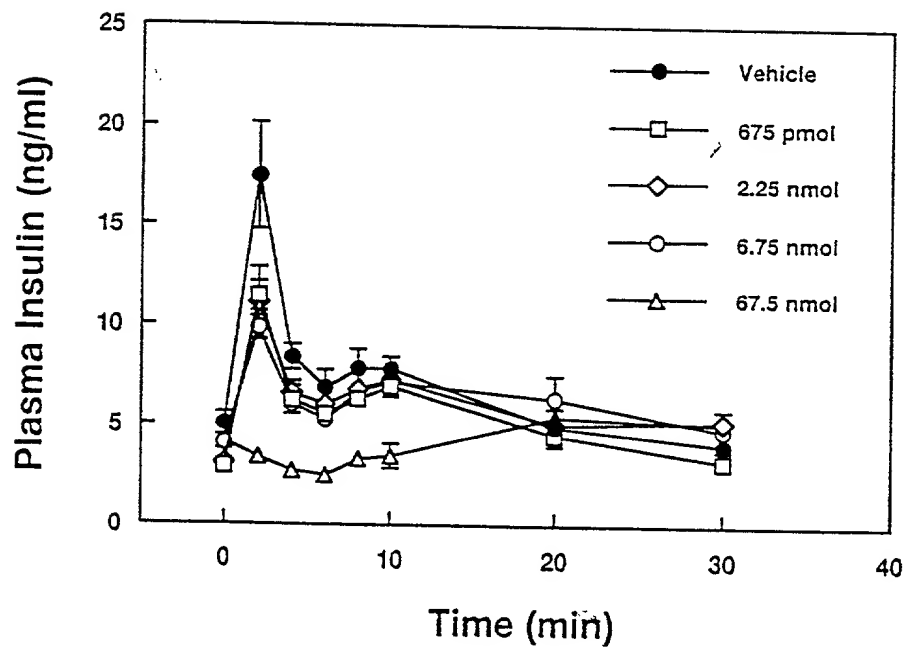
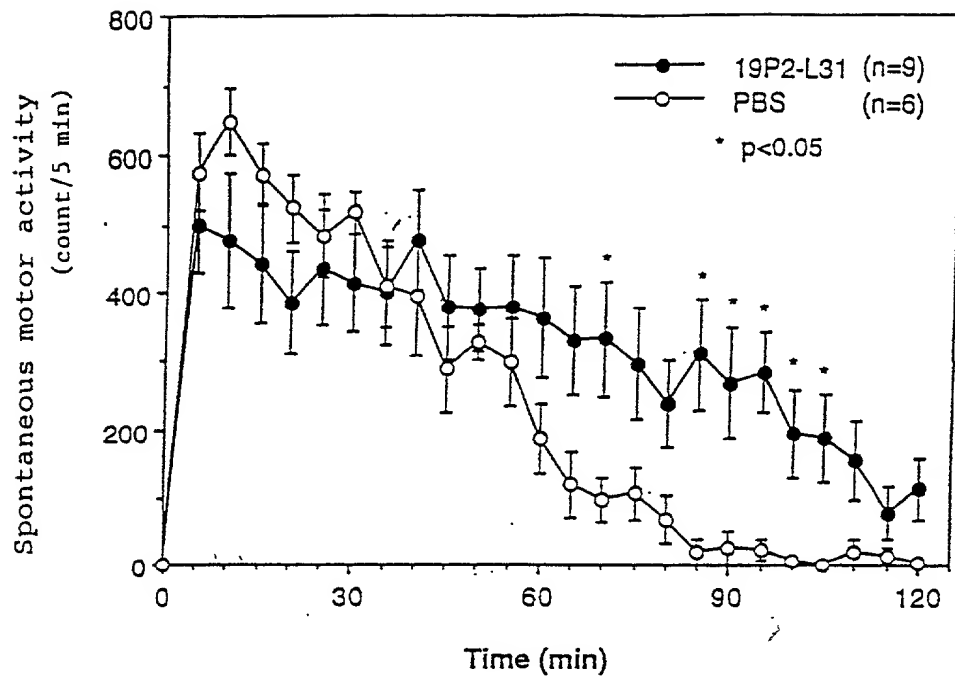




Fig. 41

(a)



(b)

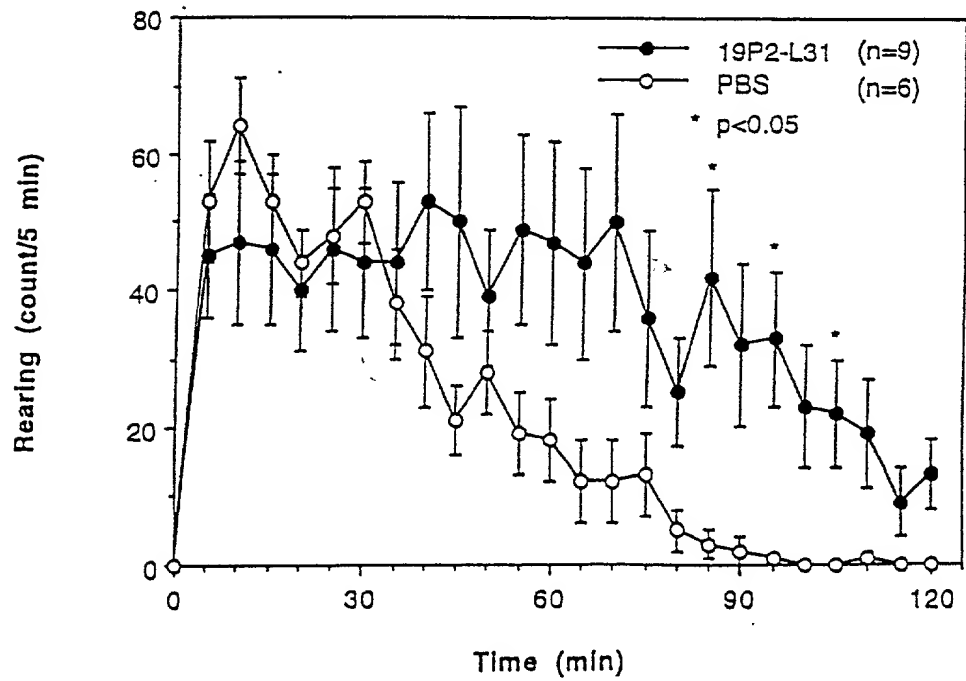
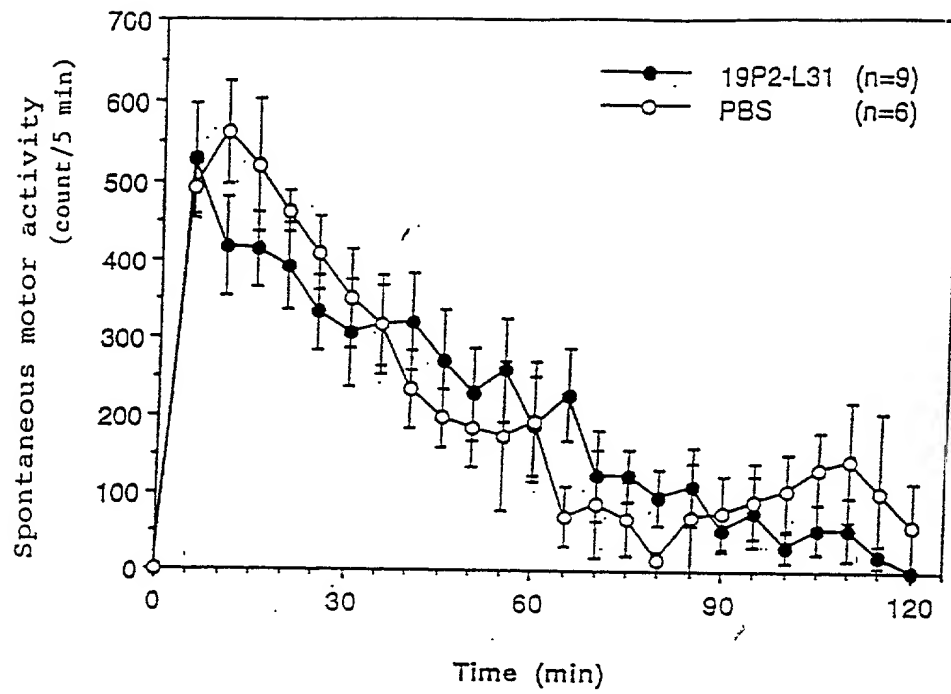


Fig. 42

(a)



(b)

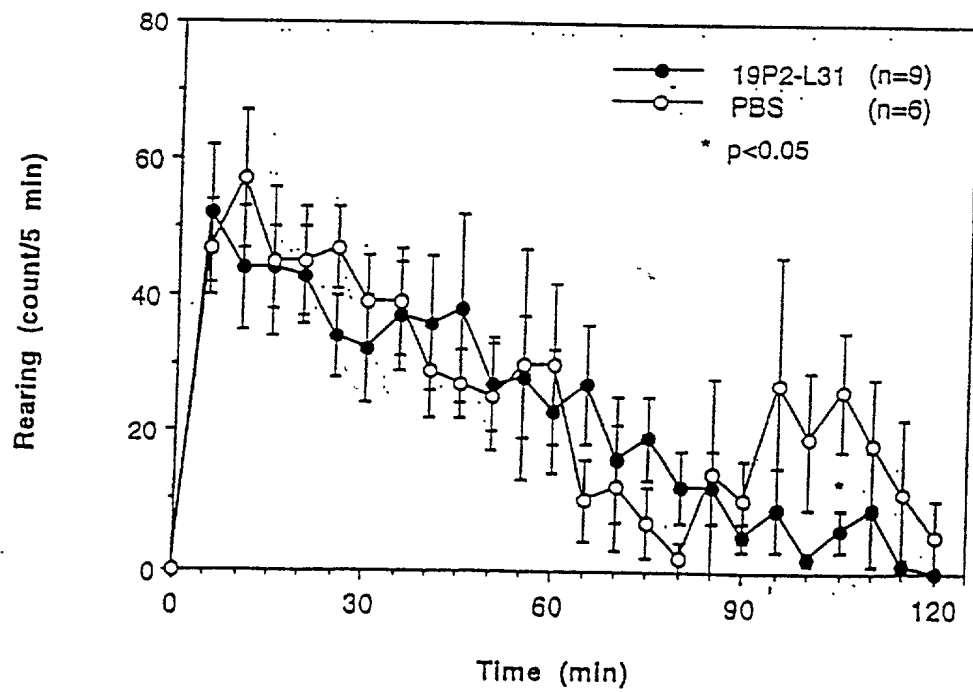
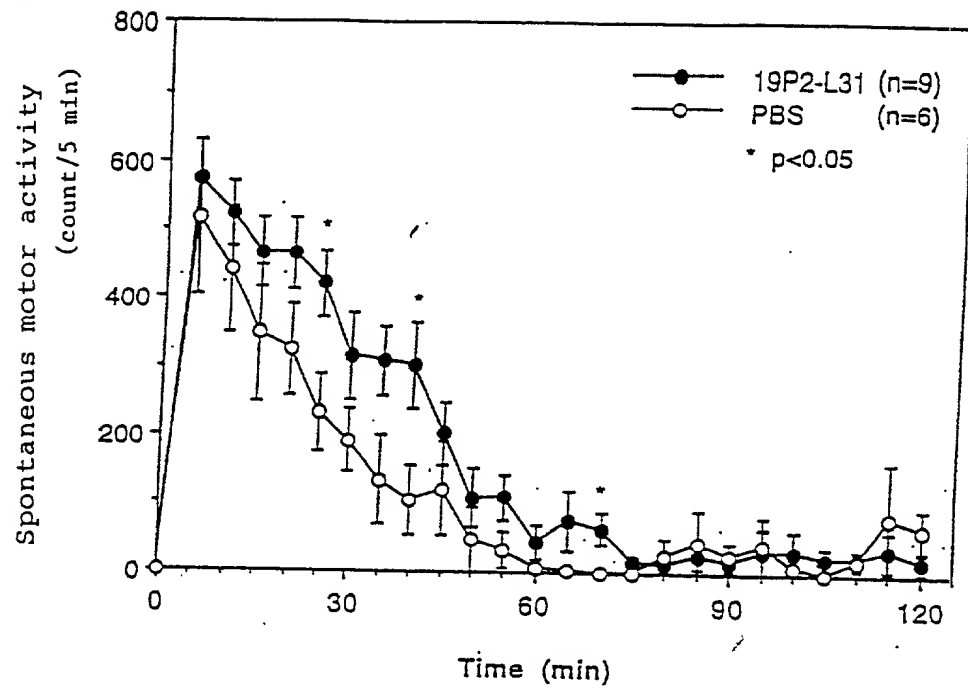


Fig. 43

(a)



(b)

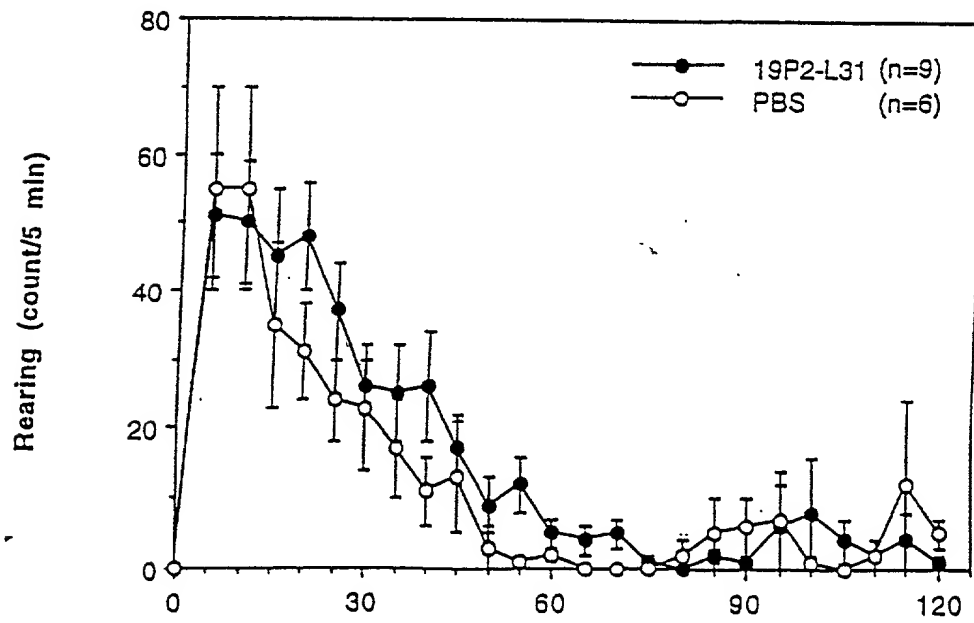
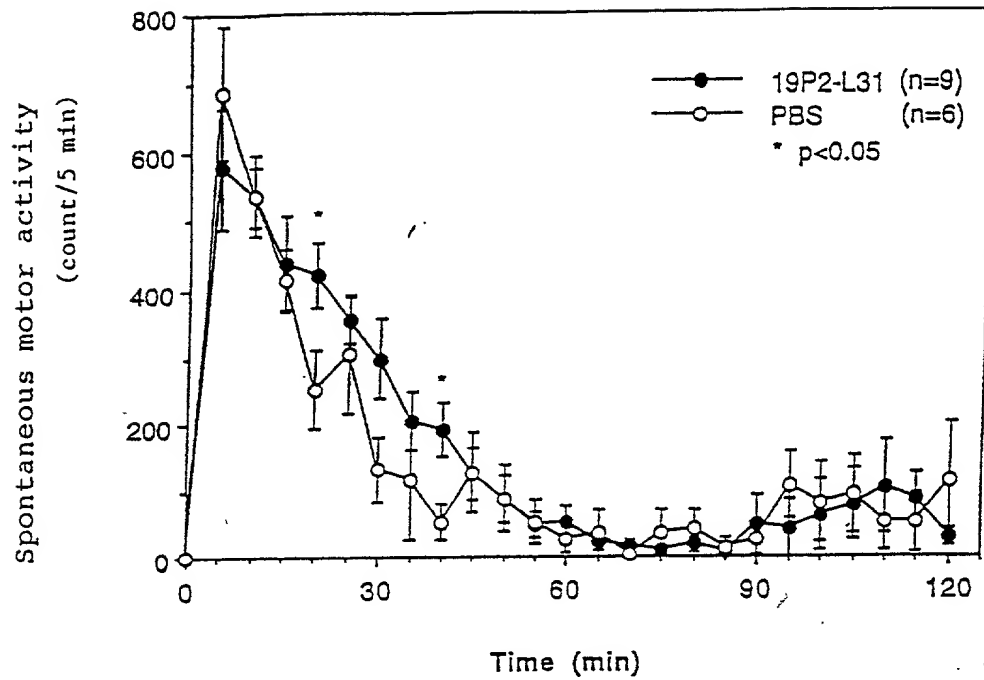


Fig. 44

(a)



(b)

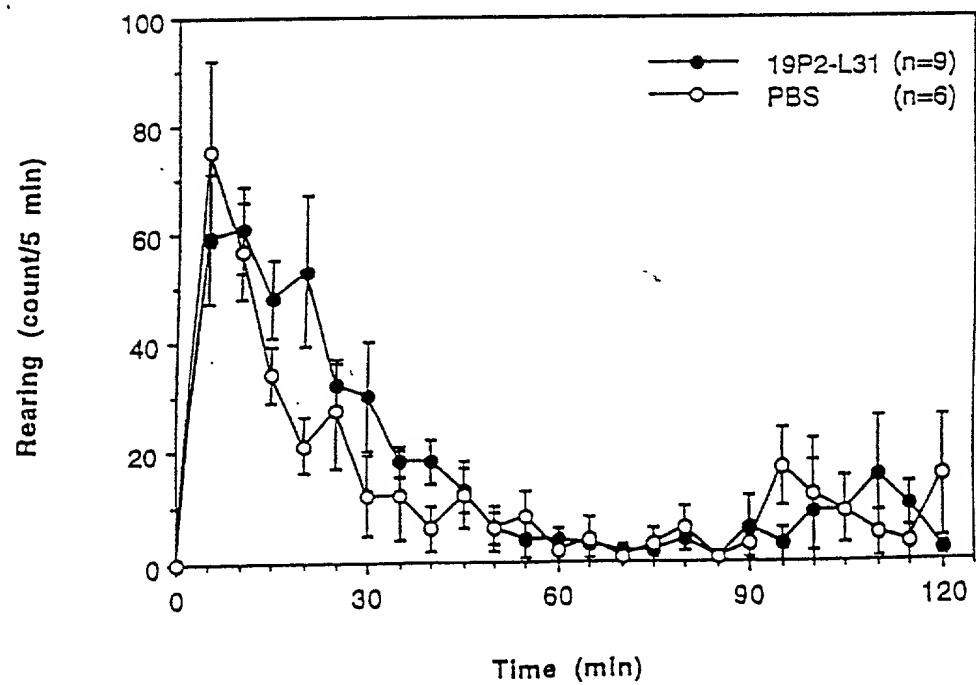


Fig. 45

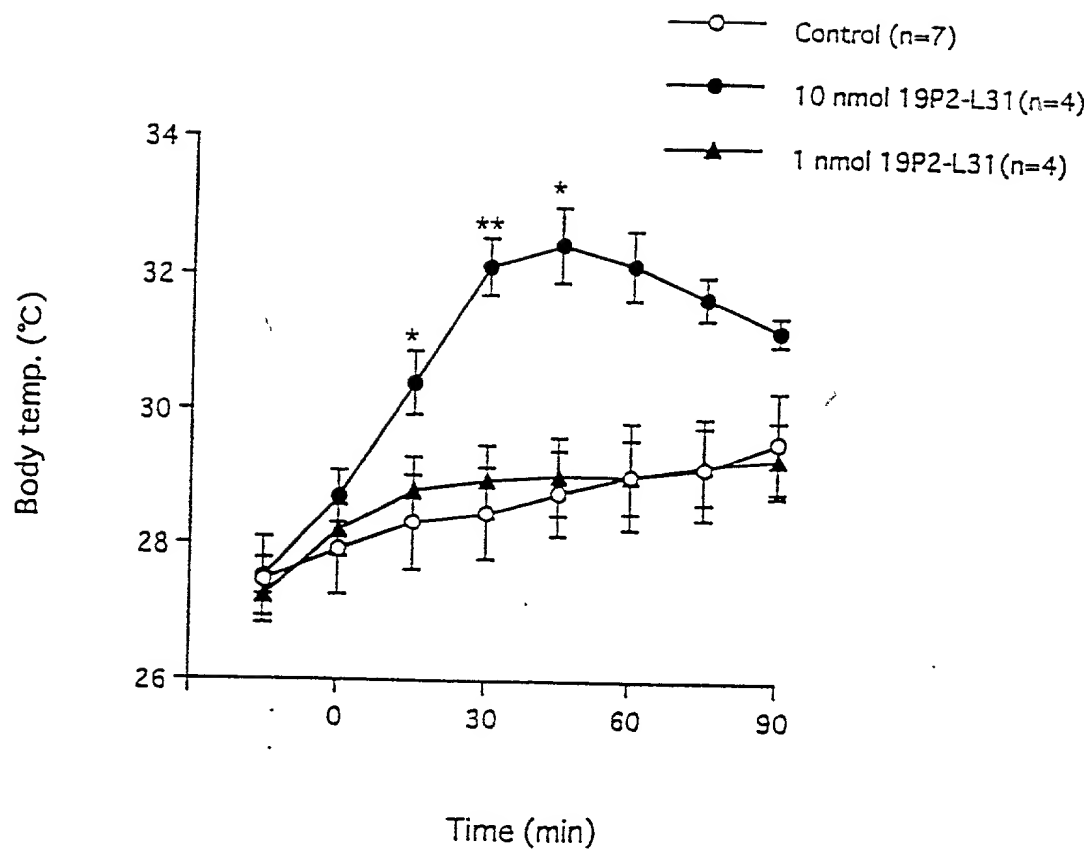
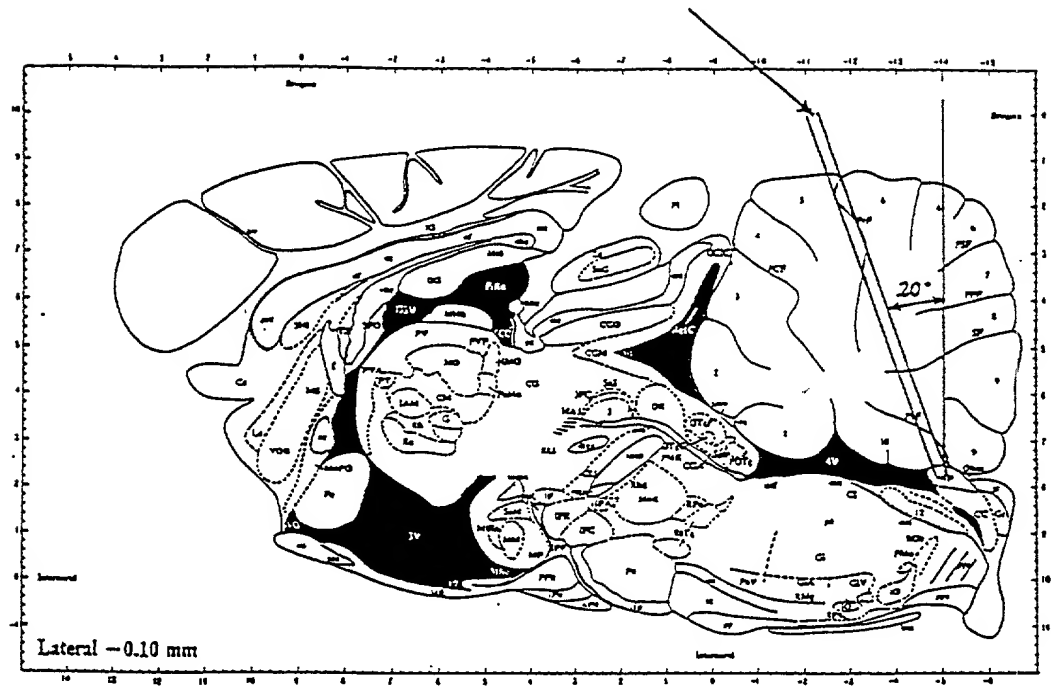
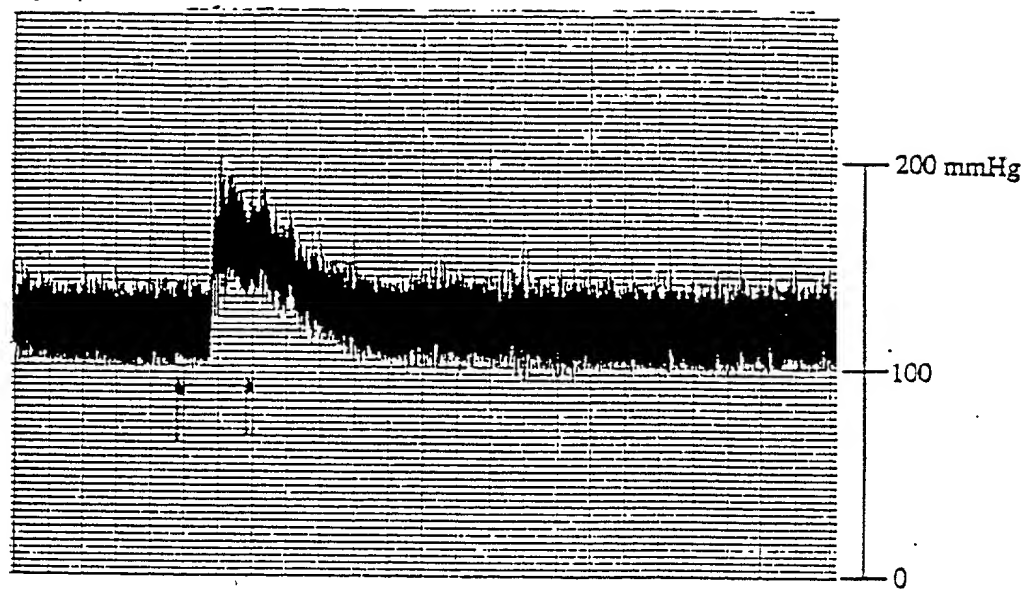


Fig. 46

micro-injection cannula



direct blood pressure



mean blood pressure

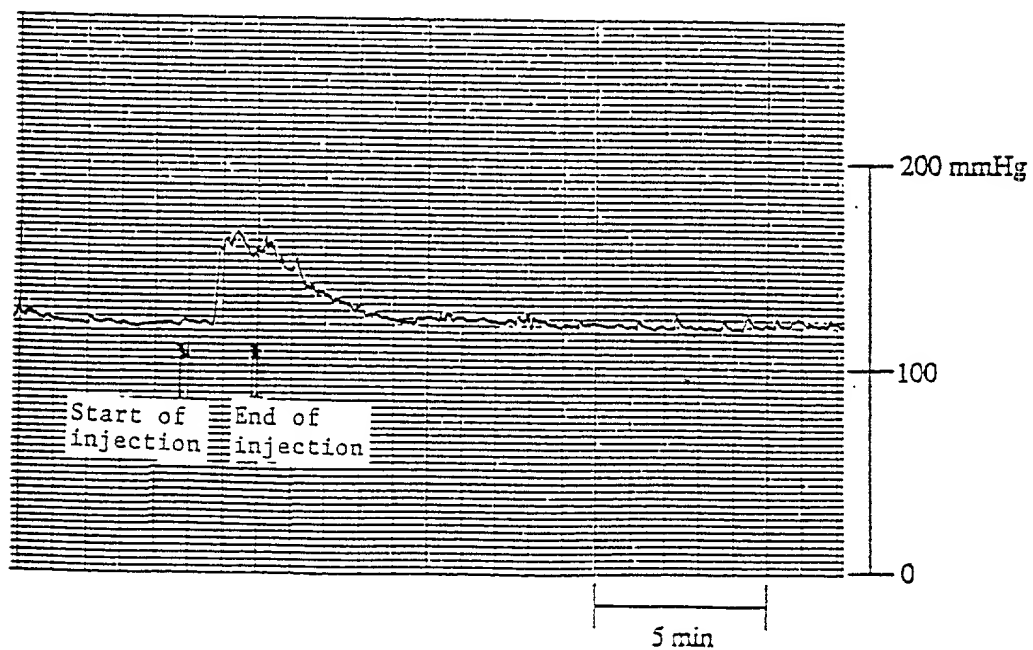


Fig. 48

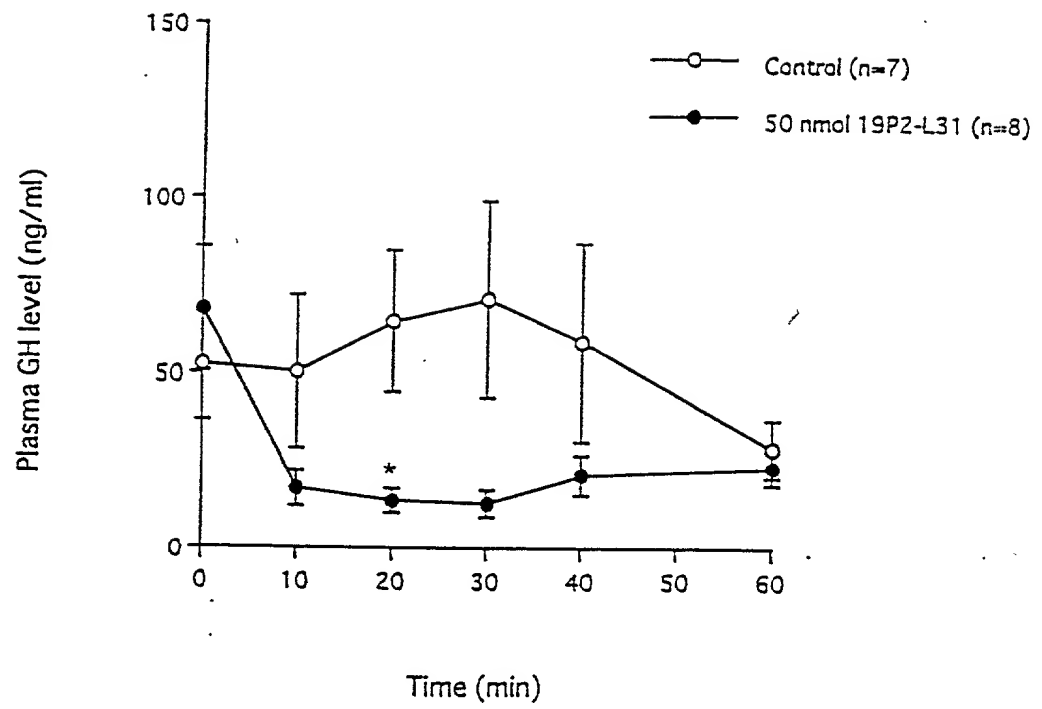




Fig. 49

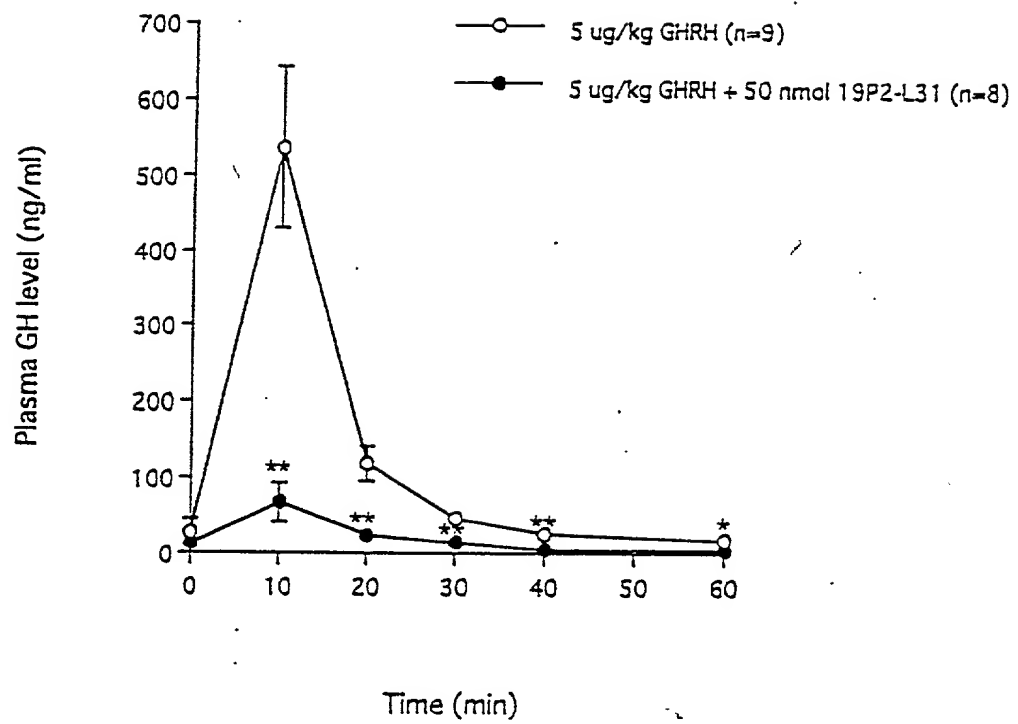


Fig. 50

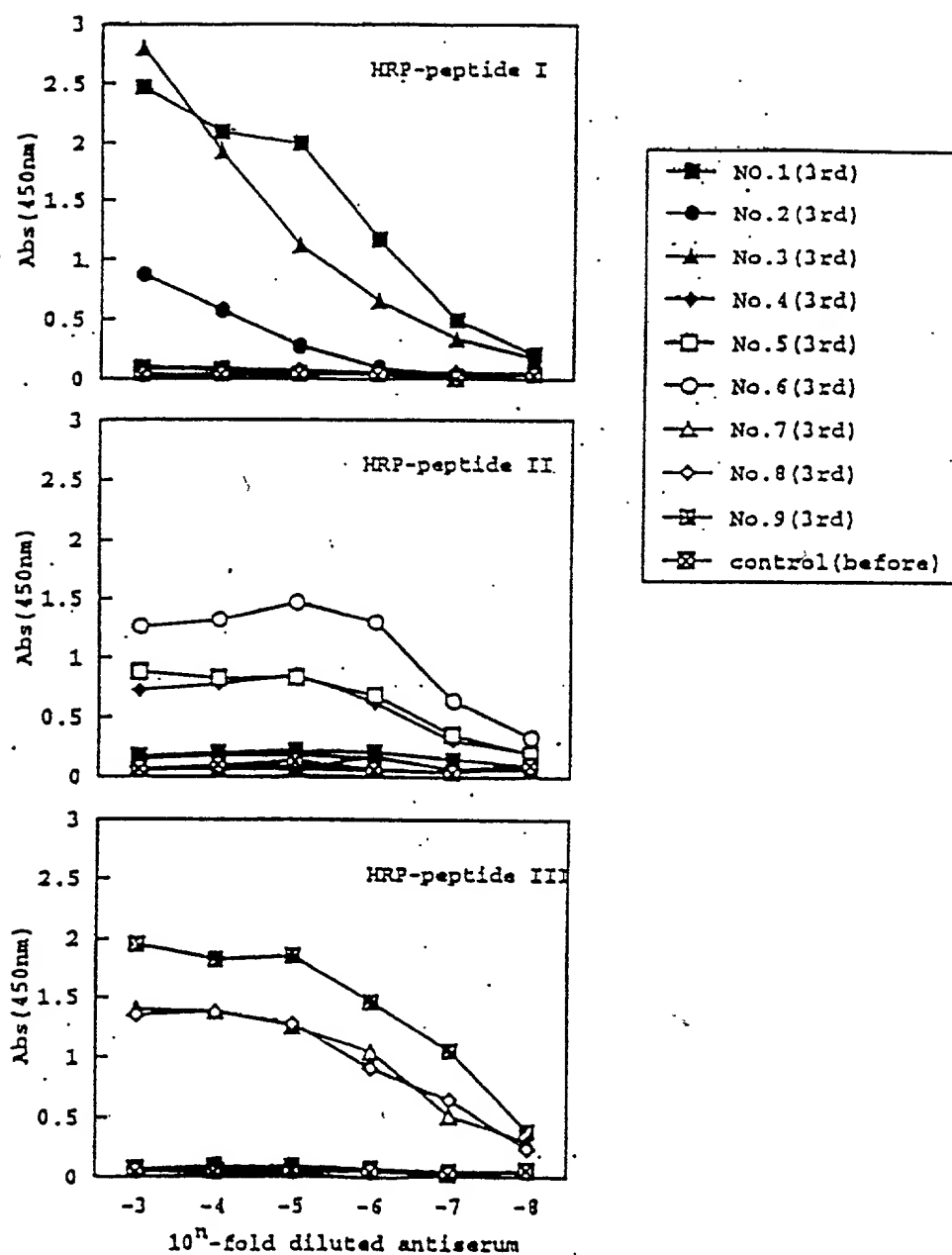
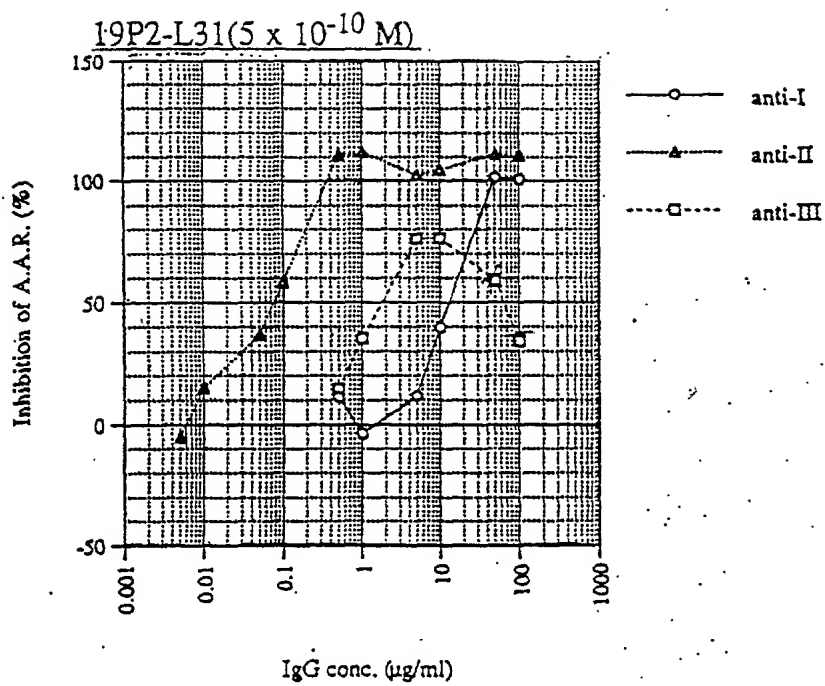


Fig. 51





**IN THE UNITED STATES PATENT AND TRADEMARK OFFICE**

In re application of: Hinuma et al.  
Serial No.: Not Yet Assigned  
[Divisional of U.S.S.N. 08/776,971  
Express Mail Label No. EL180585522US]  
Filed: Herewith  
For: POLYPEPTIDES, THEIR PRODUCTION AND USE

**Box Sequence**  
**Assistant Commissioner for Patents**  
**Washington, D.C. 20231**

**SUBMISSION OF "SEQUENCE LISTING," COMPUTER READABLE COPY,  
AND/OR AMENDMENT PERTAINING THERETO  
FOR BIOTECHNOLOGY INVENTION CONTAINING NUCLEOTIDE  
AND/OR AMINO ACID SEQUENCE**

*(check and complete this item, if applicable)*

1. ☐ This replies to the Office Letter DATED \_\_\_\_\_.

*NOTE: If these papers are filed before the office letter issues, adequate identification of the original papers should be made, e.g., in addition to the name of the inventor and title of invention, the filing date based on the "Express Mail" procedure, the serial number from the return post card or the attorney's docket number added.*

- ☐ A copy of the Office Letter is enclosed.

**IDENTIFICATION OF PERSON MAKING STATEMENT**

2. I, Christine C. O'Day  
*(type or print name of person signing below)*

state the following:

**ITEMS BEING SUBMITTED**

3. Submitted herewith is/are

*(check each item as applicable)*

- A. ☐ "Sequence Listing(s)" for the nucleotide and/or amino acid sequence(s) in this application. Each "Sequence Listing" is assigned a separate identifier as required in 37 C.F.R. § 1.821(c) and 37 C.F.R. §§ 1.822 and 1.823.
- B. ☐ An amendment to the description and/or claims, wherein reference is made to the sequence by use of the assigned identifier, as required in 37 C.F.R. § 1.821(d).
- C. ☐ A copy of each "Sequence Listing" submitted for this application in computer readable form, in accordance with the requirements of 37 C.F.R. §§ 1.821(e) and 1.824.
- D. ☒ Please transfer to this application, in accordance with 37 C.F.R. § 1.821(e), the computer readable copy(ies) from applicant's other application identified as follows:

In re application of:	Hinuma et al.		
Serial No.:	08/776,971	Group No.:	1646
Filed:	February 6, 1997	Examiner:	D. Romeo
For:	POLYPEPTIDES, THEIR PRODUCTION AND USE		

The Computer readable form(s) of applicant's other application corresponds to the "Sequence Identifier(s)" of the application as follows:

Computer Readable Form (other application)	"Sequence Identifier" (this application)
1-140	1-140

*NOTE: "If the computer readable form of a new application is to be identical with the computer readable form of another application of the applicant on file in the Office, reference maybe made to the other application and computer readable form in lieu of filing a duplicate computer readable form in the new application. The new application shall be accompanied by a letter making such reference to the other application and computer readable form, both of which shall be completely identified." 37 C.F.R. 1.821(e).*

- E. ☐ A statement that the content of each "Sequence Listing" submitted and each computer readable copy are the same, as required in 37 C.F.R. § 1.821(g).
- ☐ Because the statement is not made by a person registered to practice before the Office, the Statement is verified as required in 37 C.F.R. § 1.821(b).
- F. ☐ Because this submission is made in fulfilling the requirement under 37 C.F.R. § 1.821(g), a statement that the submission includes no new matter.
- ☐ Because the statement is not made by a person registered to practice before the Office, the statement is verified, as required in 37 C.F.R. § 1.821(g).

[illegible]

4. I hereby state:

(complete applicable item A and/or B)

- A. ☒ Each computer readable form submitted in this application, including those forms requested to be transferred from applicant's other application, is the same as the "Sequence Listing" to which it is indicated to relate.
- B. ☒ All papers accompanying this submission, or for which a request for transfer from applicants' other application, introduce no new matter.

## STATUS

5. Applicant is

- [ ] a small entity. A statement:  
[ ] is attached.  
[ ] was already filed.  
[X] other than a small entity.

## EXTENSION OF TERM

6.

*NOTE: "Extension of Time in Patent Cases (Supplement Amendments) If a timely and complete response has been filed after a Non-Final Office Action, an extension of time is not required to permit filing and/or entry of an additional amendment after expiration of the shortened statutory period.*

*If a timely response has been filed after a Final Office Action, an extension of time is required to permit filing and/or entry of a Notice of Appeal or filing and/or entry of an additional amendment after expiration of the shortened statutory period unless the timely-filed response placed the application in condition for allowance. Of course, if a Notice of Appeal has been filed within the shortened statutory period, the period has ceased to run." Notice of Dec.10, 1985 (1061 O.G. 34-35).*

*NOTE: See 37 C.F.R. 1.645 for extensions of time in interference proceedings and 37 C.F.R. 1.550(c) for extensions of time in reexamination proceedings*

7. The proceedings herein are for a patent application and the provisions of 37 C.F.R. 1.136 apply.

(complete (a) or (b) as applicable)

- (a) [ ] Applicant petitions for an extension of time under 37 C.F.R. 1.136 (fees: 37 C.F.R. 1.17(a)(1)-(4)) for the total number of months checked below:

	Extension (months)	Fee for other than small entity	Fee for small entity
[ ]	one month	\$110.00	\$ 55.00
[ ]	two months	\$400.00	\$ 200.00
[ ]	three months	\$950.00	\$ 475.00
[ ]	four months	\$1,510.00	\$ 755.00

Fee \$ \_\_\_\_\_

If an additional extension of time is required, please consider this a petition therefor.

(check and complete the next item, if applicable)

- [ ] An extension for \_\_\_\_\_ months has already been secured, and the fee paid therefor of \$ \_\_\_\_\_ is deducted from the total fee due for the total months of extension now requested.

Extension fee due with this request \$ \_\_\_\_\_

**OR**

- (b) [X] Applicant believes that no extension of term is required. However, this conditional petition is being made to provide for the possibility that applicant has inadvertently overlooked the need for a petition and fee for extension of time.

### **FEE PAYMENT**

8. [ ] Attached is a check in the sum of \$ \_\_\_\_\_.

- [ ] Charge Account No. \_\_\_\_\_ the sum of \$ \_\_\_\_\_.

A duplicate of this transmittal is attached.

### **FEE DEFICIENCY**

9.

*NOTE: If there is a fee deficiency and there is no authorization to charge an account, additional fees are necessary to cover the additional time consumed in making up the original deficiency. If the maximum, six-month period has expired before the deficiency is noted and corrected, the application is held abandoned. In those instances where authorization to charge is included, processing delays are encountered in returning the papers to the PTO finance Branch in order to apply these charges prior to action on the cases. Authorization to charge the deposit account for any fee deficiency should be checked. See the Notice of April 7, 1986, 1065 O.G. 31-33.*

10. [X] If any additional extension and/or fee is required, charge Account No. 04-1105.



**SIGNATURE(s)**

Christine C. O'Day

(type or print name of person signing statement)

Christ C. O'Day

Signature

May 23, 2000

Date

Dike, Bronstein, Roberts & Cushman, LLP

130 Water Street

P.O. Address of Signatory

Boston, MA 02109

(If applicable)

Tel. No.: (617) 523-3400

Reg. No. 38,256

☐ Inventor

☐ Assignee of complete interest

☒ Person authorized to sign on behalf of  
assignee

☐ Practitioner of record

☐ Filed under Rule 34(a)

☐ Registration No. \_\_\_\_\_

☐ Other \_\_\_\_\_

(specify identity of person signing)

(complete the following, if applicable)

\_\_\_\_\_  
(type name of assignee)

\_\_\_\_\_  
Address of assignee

\_\_\_\_\_  
Title of person authorized to sign on behalf of assignee

A "STATEMENT UNDER 37 C.F.R. 3.73(b)" is attached.

Assignment recorded in PTO on \_\_\_\_\_

Reel \_\_\_\_\_ Frame \_\_\_\_\_

**SIGNATURE OF PRACTITIONER**

Reg. No.

(type or print name of practitioner)

Tel. No.: (    )

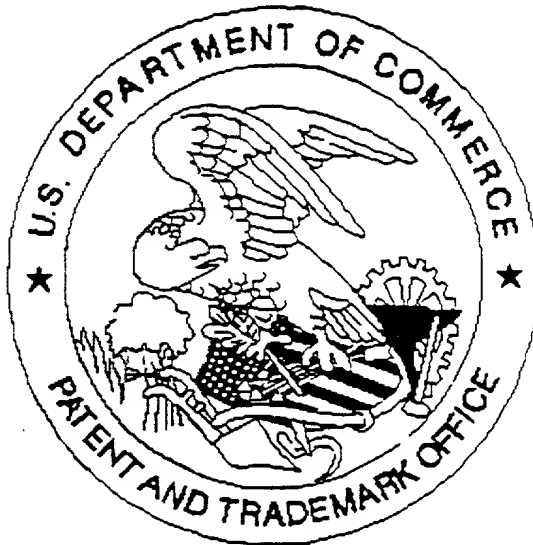
P.O. Address

Customer No.:

#118272

[illegible]

United States Patent & Trademark Office  
Office of Initial Patent Examination -- Scanning Division



Application deficiencies were found during scanning:

☐ Page(s) \_\_\_\_\_ of no Declaration were not present  
for scanning. (Document title)

☐ Page(s) \_\_\_\_\_ of \_\_\_\_\_ were not present  
for scanning. (Document title)

☐ Scanned copy is best available.